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Proceedings of the

1964 TECHNICAL SESSION

ON

CANE SUGAR REFINING RESEARCH

Held at New Orleans, Louisiana
November 9 and 10, 1964

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Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE

PREFACE

The 1964 Technical Session on Cane Sugar Refining Research was held November 9 and 10, 1964, at the Fontainebleau Motor Hotel in New Orleans, La. This conference was sponsored jointly by the Southern Utilization Research and Development Division of USDA's Agricultural Research Service and the Cane Sugar Refining Research Project, Inc., to encourage research in cane sugar refining. Technical representatives from many of the sugar refineries of the United States, including Hawaii, and from England, Canada, and Australia attended.

The program was developed by Frank G. Carpenter, Project Leader, and Bruno H. Wojcik, Assistant Director, under the guidance of C. H. Fisher, Director, with the cooperation of staff members and advisers representing the sugar refining industry.

These proceedings report the statements presented by the various speakers during the conference and give an account of the discussions following.

Numbers in parentheses refer to literature cited by the author. The figures and tables are reproduced essentially as they were supplied by the writer of each paper.

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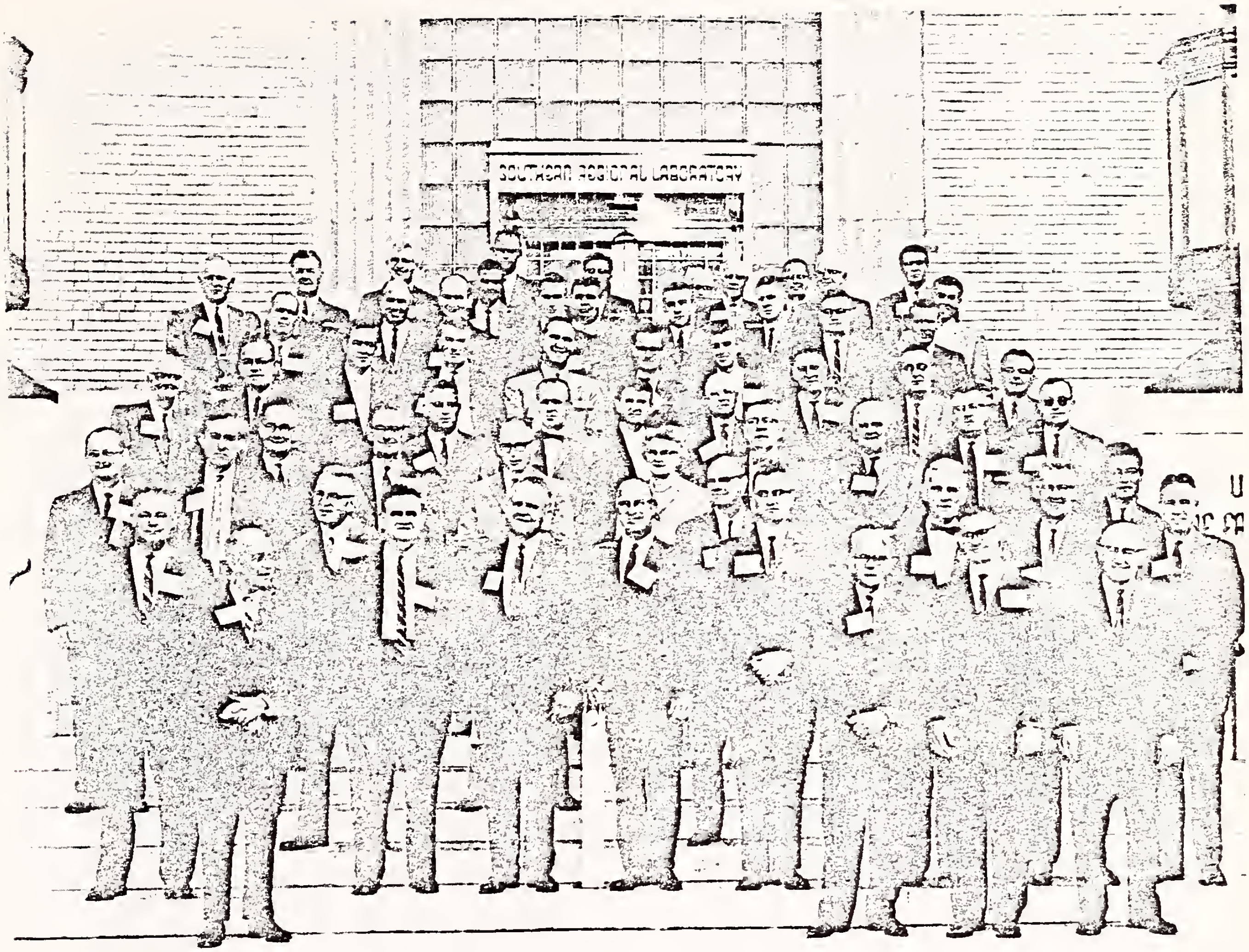

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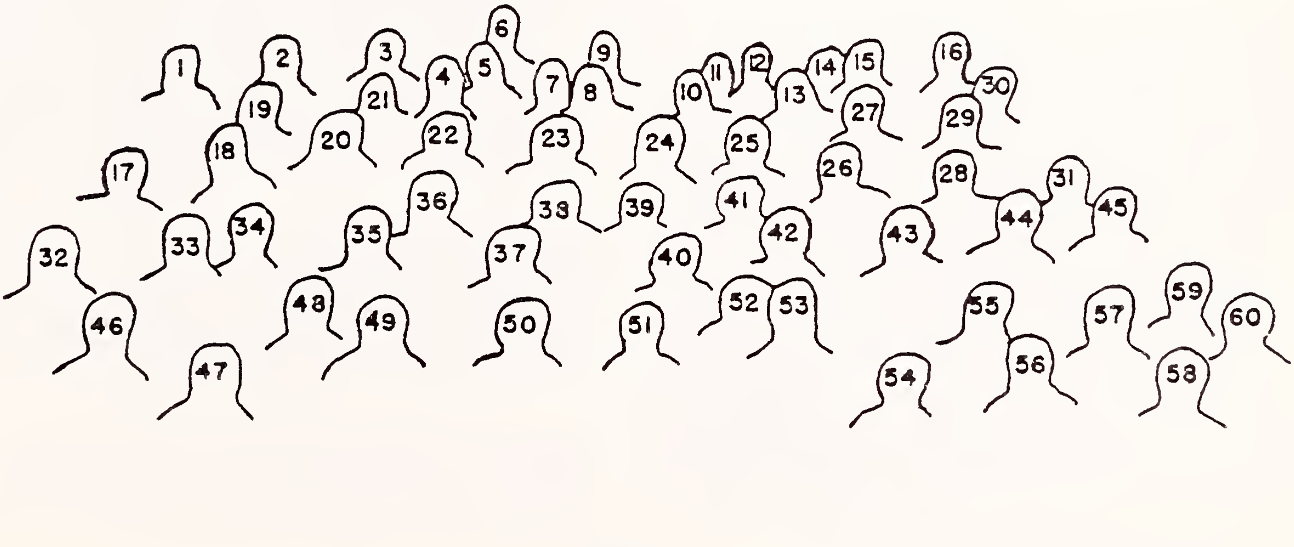

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OPENING SESSION: F. G. Carpenter, General Chairman

The 1964 Technical Session on Cane Sugar Refining Research was called to order by General Chairman Frank G. Carpenter. Dr. Carpenter then introduced Dr. C. H. Fisher, Director of the Southern Utilization Research and Development Division.

WELCOME

by

C. H. Fisher, Director

Southern Utilization Research and Development Division

It is my pleasure and honor to welcome this splendid audience to the 1964 Technical Session on Cane Sugar Refining Research. Dr. Fred R. Senti, Deputy Administrator of ARS, joins me in extending greetings and best wishes to each of you personally and for the success of the Technical Session. He regrets that he can't be present.

I would like to mention several impressive characteristics of the Technical Session and the cooperative project on sugar refining. First, I think the international aspect is impressive. Sugar is an international crop, and the Cane Sugar Refining Research Project receives international support. The participants in this Technical Session are from several different countries, including Australia on the other side of the globe. It is impressive also that the research in question is cooperative in nature. We are grateful because the Southern Division is a member of the cooperative effort. I wish to address my expression of gratitude particularly to the officers of the Cane Sugar Refining Research Project, President, E. D. Gillette; Treasurer, W. A. Bemis; and Project Director, Frank G. Carpenter. It is evident that the research of the Cane Sugar Refining Research Project supplements very well the work of other groups. Indeed, without the research of the Cane Sugar Refining Research Project there would be a serious gap in the overall sugar research program.

With respect to sugar and the USDA, it is interesting that the first investigations undertaken by the USDA's Bureau of Chemistry--when it was established about 100 years ago--were concerned with sugar.

Sugar is not the only commodity being investigated by the Southern Division. In addition, we do processing and utilization research on several other Southern crops, the principal ones being cotton, cottonseed, peanuts, fruits, and vegetables, and naval stores. During its approximately 23 years of existence, the Southern Division has provided a tremendous amount of research information--much of it basic--in about 3,000 publications and patents. About 100 commercialized developments have stemmed from--or have been substantially aided by--the research of the Southern Division. Because most of the research of the Division is done cooperatively with other organizations, credit for research successes is properly shared with them.

The major commercialized developments include: Frozen citrus concentrates, wash-wear cotton products, stretch cotton products, flame resistant cottons, improved cottonseed meal, and fresh pasteurized pickles. The total value of these developments--in terms of dollar value of the products manufactured--amounts to billions of dollars. The taxes resulting from these developments and going to the U.S. Treasury Department amount to many millions of dollars annually.

I wish to thank everyone who has contributed, or who will contribute, to the success of the 1964 Technical Session, including: the officers, members and staff of the Cane Sugar Refining Research Project; the General and Session Chairmen, Frank G. Carpenter, Elliott P. Barrett, Kenneth R. Hanson, and Henry Gerstner; the Program Committee, Frank G. Carpenter, V. H. McFarlane, and L. F. Martin; the speakers and participants in the Technical Session; Dr. Bruno H. Wojcik, and many members of our staff, including L. W. Mazzeno, Jr., and Beatrice A. Sharar; R. M. Persell, V. R. Bourdette, and Marie A. Jones.

FIRST SESSION: E. P. Barrett, The Baugh Chemical Co., Chairman

DETERMINATION OF SUITABILITY OF GRANULATED SUGAR FOR BEVERAGES BY POLAROGRAPHY

by
Raymond D. Moroz and Yigal Yankelevits
Applied Sugar Laboratories, Inc.
(Division of SuCrest Corp.)
Brooklyn, N. Y.

(Presented by Yigal Yankelevits)

Floc is a term describing a precipitate which is occasionally found in acidified carbonated beverages, bottlers' concentrates, and pharmaceutical syrups. Its origin has been traced to primarily three sources: 1) The use of impure water in the beverage plant; 2) biological activity; and 3) commercial beet and cane sugars.

Early investigation of floc was initially conducted in the beet industry and had been directed toward predicting whether a particular sugar would flocculate. Accordingly, tests were divised whereby a sterile aqueous solution of sugar, usually at 50 Brix, was acidified with the addition of citric, phosphoric, or hydrochloric acid to pH's between 1.8 and 4.0 and allowed to stand over a period of time, 1 hour to 10 days. The formation of a well-defined flocculent-type precipitate would be indicative of a floc-forming beet sugar. Another approach was based on the addition of a cationic surface active agent (quaternary anion) until maximum turbidity was attained, i.e., complete coagulation of negatively charged colloids. The application of these floc tests to cane sugar was found to be inconclusive.

Through the efforts of Eis and coworkers (1) and Walker and Owens (2), a sugar beet glycoside and derivative, also called saponin, was discovered to be an important contributing substance to the formation of floc when the sugar is derived from beet. On the other hand, according to the work of Stansbury and Hoffpauir (3), floc derived from cane sugar consisted of starch, lipids or waxes, protein, silicates, and decolorizing carbon.

Based on the floc saponin relationship, Walker (4) formulated a quick chemical procedure for determining saponin in refined beet sugar. At the same time, he pointed out that no saponins were found in cane sugar. Later, Hibbert and others (5) applied polarography in measuring the floc tendency of a sugar. In 1963, Schiweck (6) quantitatively related polarographic measurements with saponin quantity. Both Hibbert and Schiweck employed the technique suggested first by Sandera and Zimmermann (7) and later refined by Vavruch (8), that is, measurement of the suppression of the oxygen maximum in a sugar solution containing a supporting electrolyte.

At this point it is essential that the principles of polarography and some of the associated terms are explained. Polarography is an outgrowth of the unique properties exhibited by an electrolytic cell consisting of a large, difficultly polarized reference electrode, such as a pool of mercury; a small readily polarized electrode formed by a succession of mercury drops emerging from the end of a glass capillary tube; and an electrolytic solution containing trace concentrations of an electro-reducibel or electro-oxidizible material, and a much greater concentration of a less readily electrolyzed ion. When a potential, changing in value at a known fixed rate, is impressed across such a cell and the resulting current is plotted as a function of the applied voltage, there is obtained a characteristic curve which rises to a limiting value that is simply and directly related to the concentration of the reacting material and whose inflection point is located at a voltage range characteristic of the particular material. The current flowing through the dropping mercury electrode (DME) is basically composed of four types of current: 1) diffusion, 2) adsorption, 3) migration, and 4) residual.

The diffusion current describes the manner in which electroactive materials reach the DME from the solution. When the decomposition potential of a reducible ion is attained, there will be plating out on the mercury surface with subsequent formation of a concentration gradient causing the reducible ion in the solution to diffuse into the mercury solution interface. In analytical determinations, it is the diffusion current which is normally measured. The rate of diffusion is proportional to the reducible ion concentration in the bulk of the solution. When this rate becomes constant, a limiting current is reached.

Adsorption current is the least understood of the four currents because no one explanation has been satisfactory in accounting for all of its peculiarities. One of these peculiarities is the abnormal increase in current with voltage to a critical point or maximum whereupon a very small increase in voltage results in a sudden drop of current. The suppression of the maximum exhibited by the reduction of oxygen in a solution of inert electrolyte by surface active materials is an example of adsorption current. In quantitative polarographic analysis, it is customary to add a suppressor, a substance more adsorbable at the mercury solution interface than oxygen, to eliminate such a maximum. Measuring the floc tendency of a sugar makes use of the displacement of oxygen ions by a suppressor or any material having surface activity.

If electroactive substances are placed in an electrical field, the positive ions will be attracted to the negative mercury drops under the influence of an electrical potential difference between the mercury drop and solution. The migration of oppositely charged ions to the DME, in this manner is called the migration current.

The interface between the mercury droplet and an inert electrolyte, that is, an electrolyte neither reduced nor oxidized at the DME, acts as a condenser. Each drop will be charged when connected across a voltage source. The current intensity required to charge each drop is approximately proportional to the applied potential. This produces a linearly increasing current known as the residual current. An interesting phenomenon associated with residual current is the electrocapillary curve of mercury, a relationship between the interfacial tension and applied voltage. When current is applied to the polarographic cell, the DME immediately polarizes and as the potential increases so does the interfacial tension until a maximum is reached at which point the DME is electrically uncharged. At this maximum point, also called the isoelectric point of mercury, the drop time of the mercury is at its highest level. Those substances that can affect the shape of the electrocapillary curve, i. e., the position of the maximum along the voltage axis and the maximum value of the interfacial tension (also drop time) are called capillary active. Sucrose is one of the capillary active substances and will give a definite curve. Impurities associated with sugar solutions have been found capillary active and will also alter this curve. Since the chemical composition of floc varies and the physicochemical mechanisms by which it is formed are not known, a standard curve prepared with known concentrations of floc cannot be made. Therefore, in order to verify the assumption that electrocapillary active substances are responsible for floc, a reference floc test had to be established. The method chosen was the preparation of a modified carbonated beverage containing the questionable sugar.

Beverage Test

1. Sterilize a 7 oz. soda bottle in an autoclave under 15 p. s. i. pressure and 120° C. steam for 15 minutes. Cool bottle.

2. Add to bottle 21 g. of dry sugar and 25 ml. of distilled water that has been filtered through a 0. 45 micron membrane filter. If a liquid sugar is being tested, add 21 g. of dry sugar equivalent and accordingly decrease the amount of water.

3. After the sugar is dissolved, heat to 85° C. in a water bath and maintain for 3 minutes. Cool to room temperature.

4. Add 2 ml. of a citrate buffer consisting of 0.076 g. of sodium citrate and 0.25 g. of hydrous citric acid. Mix well. The citrate buffer solution should have been previously filtered through a 0.45 micron membrane filter.

5. Cool mixture to about 40° C. and add carbonated water to a designated level. The total volume amounts to approximately 207 ml. Carbonated water is prepared by using a home-use carbon dioxide cartridge device (Sparklett). A membrane filter in a line holder is placed on the outlet tube within the vessel containing carbonated water.

6. The bottle is quickly capped with a capping device. The caps must be previously sterilized by inserting them in boiling water for 5 minutes.

7. Observation is made daily for 14 days. A microscopic light illuminator and gentle swirling of the bottle will aid in observing any formation of a white flocculent precipitate. Sometimes a light fluffy floc forms which tends to remain suspended above the bottom of the bottle. This type is the result of biological activity which should be removed and examined microscopically for microbiological cells, pseudomycelium, mycelia, ascospores, and ooidospores.

With the beverage test as a reference method, we proceeded to establish the conditions needed in obtaining polarograms that could be related to floc. Four methods were investigated. The first method was similar to the one first suggested by Vavruch (8) on the classification of sugar by polarography. The second and third methods employed different supporting electrolytes at lower sugar densities. The fourth method involved the tracing of a polarogram without the addition of an inert electrolyte.

All the work was carried out with the Model XV Sargent Recording Polarograph. The mercury drop time, 4.5 seconds, and temperature, 25°C., remained constant. It is important that the drop time be checked prior to the tracing of the polarogram.

The polarographic cell consisted of the dropping mercury electrode as the cathode and a pool of mercury as the anode. The mercury is used only once and is then cleaned according to the procedure recommended by Muller (9). It is important that the mercury remains clean.

When assigning quantitative values to the height of the maximum, Vavruch's procedure was followed, i.e., h is the symbol given to the maximum height as measured from the highest point in the curve to the upper part of the diffusion or limiting current and is expressed in microamperes (Figure 2).

Method No. 1 - Potassium Sulfate

Concentration of Sugar, g./100ml.---26
Electrolyte---0.002 N K_2SO_4
Drop Time, sec.---4.5
Temperature, °C.---25
Full Sensitivity Range, microamps---10
Damping---0

The comparative results between the above operating conditions and the beverage test are given in Table I.

Table 1.--Method No. 1 vs. beverage test

Polarographic test h(μ A)	Number of samples beverage test	
	Floc positive	Floc negative
0 to 0.99	14	6
1.0 to 2.0	4	9
2.0 to 3.0	1	6
Greater than 4.0	0	1

The data in table 1 indicate that the use of potassium sulfate as a supporting electrolyte does not give a satisfactory picture between those sugars containing floc and those not containing floc.

It was decided that substitution of potassium sulfate with the same citrate buffer employed in the beverage test might enhance the activity of the components of floc. Also the density of the test solution was reduced to 10 Brix.

Method No. 2 - Citrate Buffer

Concentration of Sugar, g./100 ml. ---10
Electrolyte---1 ml. Citrate Buffer per 100 ml.
Citrate Buffer---0.038 g. of sodium citrate per ml. and 0.125 g. of hydrous citric acid per ml.
Drop Time, sec. ---4.5
Temperature, °C. ---25
Full Sensitivity Range, microamps---10
Damping---0 and 2

The results using citrate solution are given in table 2.

Table 2. --Method No. 2 vs. beverage test

Polarographic test h(μ A)	Number of samples beverage test	
	Floc positive	Floc negative
Less than 2.0	4	0
2.0 to 3.0	12	4
3.0 to 3.5	3	4
3.5 to 4.0	2	0
4.0 to 5.0	1	7
Greater than 5.0	0	5

The use of citrate buffer and a lower density gives a higher oxygen maximum. However, the distribution between floc positive and negative sugars and the reproducibility of the results were not up to expectations. Variations on triplicate analysis of the same sample amounted to 20 percent.

Method No. 3 - Potassium Chloride

The same conditions existing for Method No. 2 except that 0.001 N KC1 was used as the inert electrolyte.

Table 3 illustrates the relationship between the beverage test and Method No. 3.

Table 3. --Method No. 3 vs. beverage test

Polarographic test h(μ A)	Number of samples beverage test	
	Floc positive	Floc negative
Less than 5.0	16	0
5.0 to 6.0	10	14
Greater than 6.0	0	12

The correlation between the beverage test and Method No. 3 is good. In the region of 5.0 to 6.0 μ A, the normal current rise to the oxygen maximum is not always linear but shows the presence of one or more small maxima. When only one of these maxima is present and the h value is greater than 5.0, the sugar gave a negative floc result with the beverage test. (Figures 1 and 2) When two or more maxima occured, the beverage test indicated positive floc. (Figure 3) However, in order to study further the sugars having these minute maxima, we decided to analyze the polarogram of the sugar without any electrolyte. The pattern of the residual current of sucrose showed that with more than one maxima on the ascending branch of the oxygen curve, the sugar will floc.

Method No. 4 - No Electrolyte

The same operating conditions as in Method No. 2 except that no electrolyte is used.

In table 4, the comparative results between the beverage test and Method No. 4 are recorded.

Two types of polarograms are possible when no electrolyte is used in the test sugar solution.

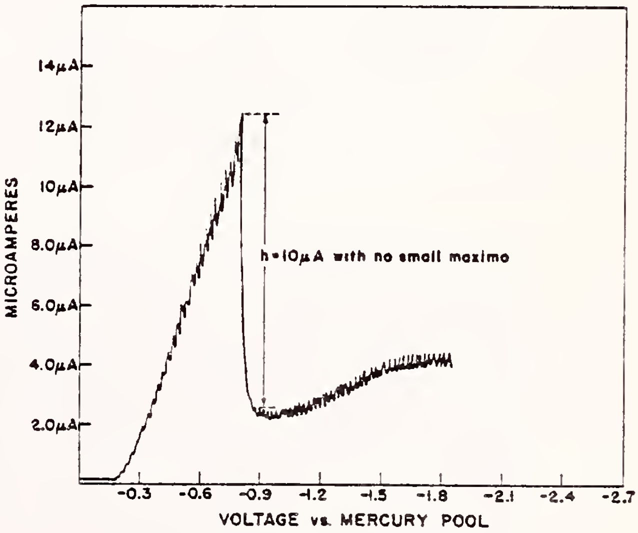

Figure 1.--Granulated sugar, floc-free, 0.001N KC1 electrolyte.

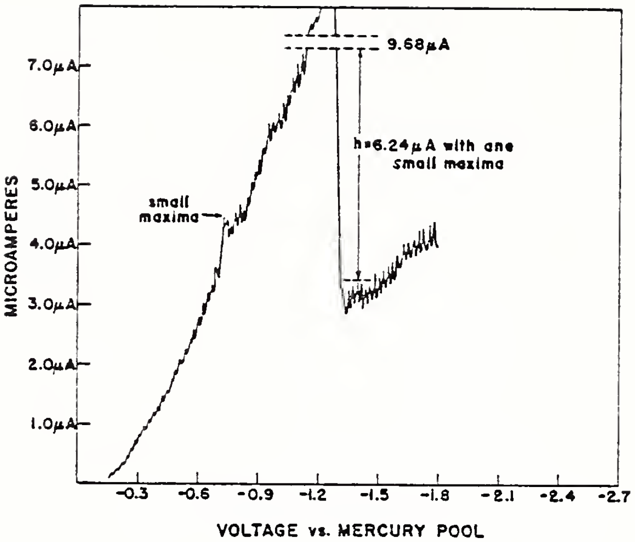

Figure 2.--Granulated sugar, floc-free, 0.001N KC1 electrolyte.

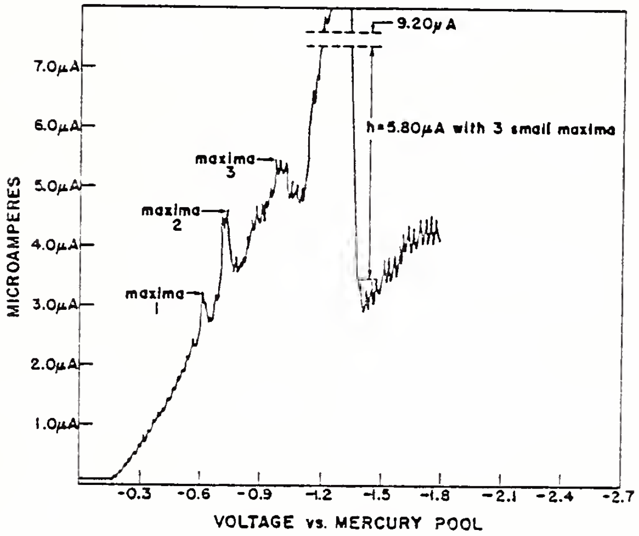

Figure 3.--Granulated sugar, floc-positive, 0.001N KC1 electrolyte.

If a standard sugar is prepared by the combined treatment of vegetable carbon, ion exchange and recrystallization, a curve will form in which the absolute slope will be less than 0.2 as plotted with the equipment used in this investigation. This corresponds to a final amperage of less than 2.0 μ A at -3.0 V. (Figure 4) The absence of electrolyte impurities will tend to increase the potential across the solution but will not permit an increase in current intensity. This results in a quantity of amperage that is allowed by the ionization constant of sucrose and water.

Table 4. --Method No. 4 vs. beverage test

Polarographic test	Number of samples beverage test	
	Floc positive	Floc negative
Showing a maximum:		
Ratio of h to Voltage less than 1	14	0
Ratio of h to Voltage greater than 1	0	12
Showing no maximum:		
μ A at -3.0 V greater than 4.0	17	0
μ A at -3.0 V less than 2.0	0	7

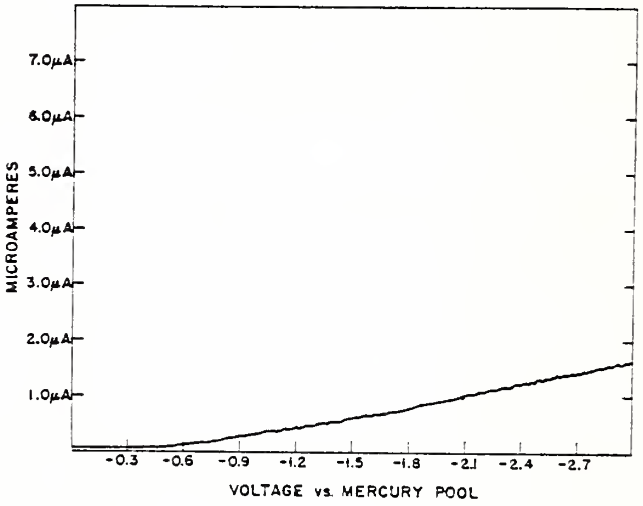

Figure 4.--Special granulated sugar, floc-free, no electrolyte.

The second type of polarogram that can be formed without any electrolyte is one in which a high maximum occurs at a voltage greater than -1.5. A small increase in nonsugar substances will increase the current intensity of the solution with rising potentials. This permits eventually an oxygen maximum to occur. As the impurities of the sugar become greater, a suppression of oxygen maximum takes place.

The maximum will decrease in proportion to the concentration of the floc components in the sugar. It has been found that if the ratio of the maximum, h, to the voltage at which this maximum occurs is greater than one microampere per volt, then the concentration of floc constituents is low enough not to cause flocculation in the beverage. (Figure 5) When the ratio is less than one, the sugar will invariably cause floc. (Figure 6 and 7) When floc producing materials are present in high concentrations, a point will be reached where no maximum occurs and the curve will reach a current intensity of greater than 4.0 μ A at -3.0 V. (Figure 8)

The elimination of inert electrolytes tends to stress the effect of the residual current. In doing this, we have accentuated the effect of any capillary active substances found in sugar. Some of these components are undoubtedly floc formers.

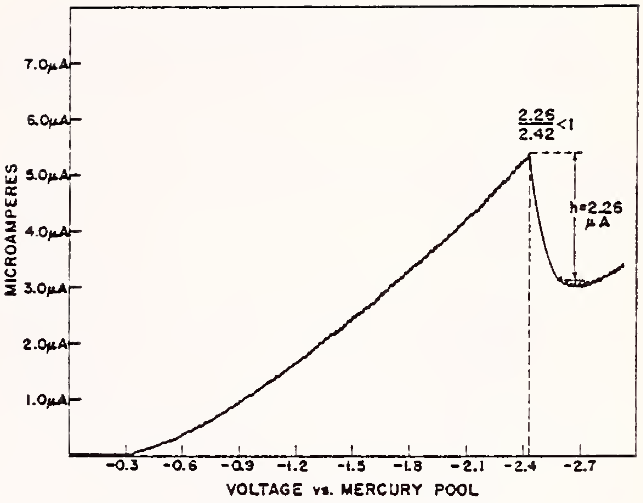

Figure 6.--Granulated sugar, floc-positive, no electrolyte.

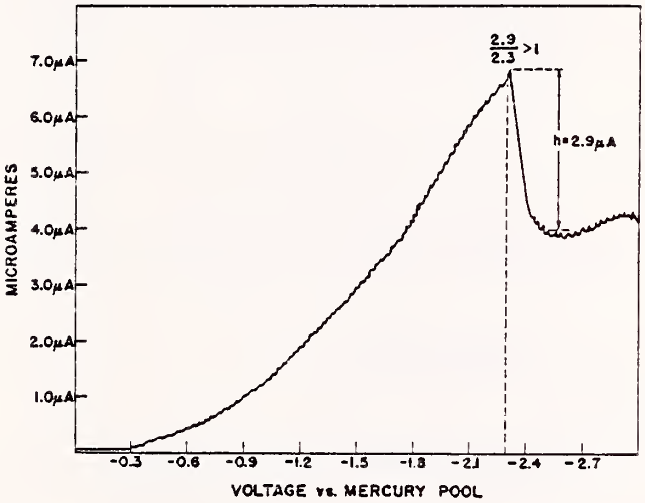

Figure 5.--Granulated sugar, floc-free, no electrolyte.

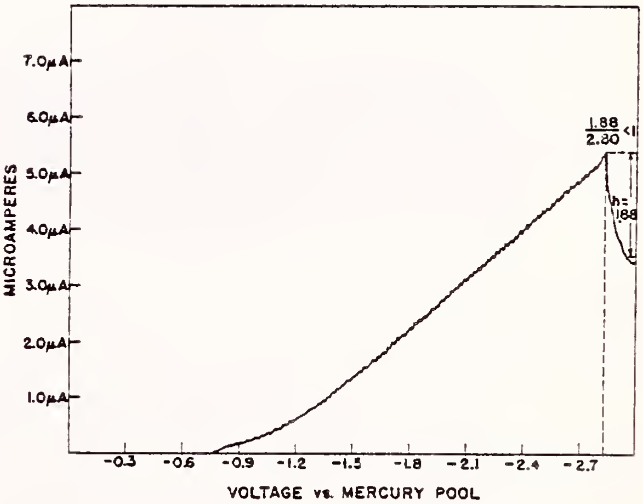

Figure 7.--Granulated sugar, floc-positive, no electrolyte.

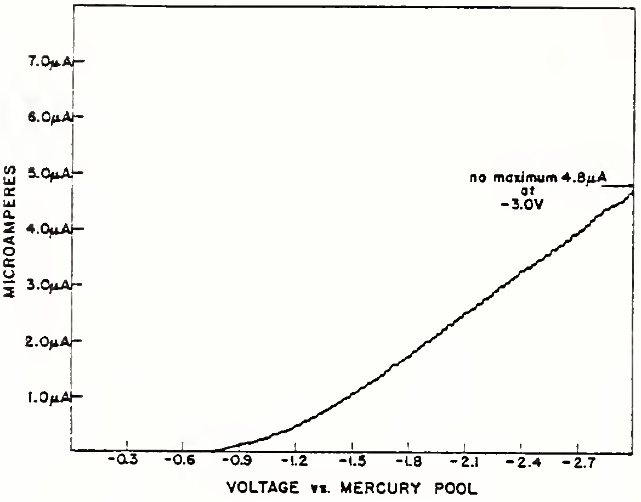

Figure 8.--Granulated sugar, floc-positive, no electrolyte.

Conclusion

The assessment of the floc components of granulated sugar can be determined by utilizing the suppression of the oxygen maximum in a 0.001 N KCl solution and/or tracing a polarogram without an electrolyte and analyzing the resulting curve.

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DISCUSSION

S. Borodkin (American): You showed a difference between some pure materials that didn't floc and a Number 3 sugar that produced floc. Do you have any examples of a Number 3 sugar that didn't floc, and can you tell the difference by polarography?

Y. Yankelevits (SuCrest): With the polarographic test there was no case using the fourth method which gave floc-positive on the polarographic and floc-negative in the bottlers' test. As I said before, this is a reliable test. The sugars used were first, second, third, and fourth strikes. As you probably all know, the first strike would be better than second and down the line. The third strike sugar used was floc-positive in the bottlers' test and it also showed floc-positive on the polarogram.

S. Borodkin: In other words, every Number 3 sugar flocced?

Raymond D. Moroz (SuCrest): We did not test all the sugars on the polarograph. There were two third strike sugars in the last year that were floc-negative, but there were about 40 or 50 that were floc-positive.

S. Borodkin: What did the polarogram show?

Raymond D. Moroz: At that time we were using the beverage test, and it showed floc-negative. We did not have a polarograph at that time to find out what it would show.

T. M. Rinehart (Atlas): Was any work done on using the polarograph on feed liquor to the pans to predict whether the strike would be floc-forming?

Raymond D. Moroz: Yes, unfortunately, when we were working with the pan feed liquor, we were using the electrolyte. In this system you get no peak without oxygen. We were adding oxygen and got erroneous conclusions. We could not make any sense out of it. We are intending to try out this scheme on liquors without any electrolyte. But we had to study some of the unusual characteristics of residual current particularly the effect of ash on residual current.

One more important factor is the concentration of the sugar solution. In the potassium sulfate treatment, we used 26 g. in 100 ml. of solution. In the potassium chloride and with citrate buffer and also with no electrolyte treatment, we used 10 g. in 100 ml. The reason for this is that sucrose is capillary active, and it will effect some of these peaks itself. To enhance the impurities, the surface-active agents, we decided to drop the density. At this point, we began to get useful results.

S. Borodkin: Will the introduction of surface-active agents such as Triton X-100 to the samples that gave the oxygen maximum cause it to disappear?

R. Moroz: We didn't try that.

J. Dowling (Refined Syrups and Sugars): I'd like to straighten out the criterion for judging floc. In the fourth method where no supporting electrolyte was used, in some cases there was a continuous slope and in other cases there was an oxygen maximum. Is it the difference between the oxygen maximum and the following minimum divided by the voltage that is the criterion, or is it the complete voltage and complete current?

Y. Yankelevits: It is the voltage at which oxygen maximum takes place divided into the amperage of the h value or difference between the oxygen maximum and the following low value.

J. Dowling: And when no maximum occurs, is it a slope of less than 0.2 that is required to indicate no floc?

Y. Yankelevits: A slope of less than 0.2 as we measure it with our apparatus is the criterion. We haven't mentioned partial invert and things of that nature because we haven't fully investigated them. For instance, partial invert is floc-free. However, the polarogram obtained does not indicate floc-free as we see it with dry sugar. Therefore, we haven't discussed it.

J. Dowling: Do you know why at times you get a slope and at other times you get an oxygen maximum?

Y. Yankelevits: Yes, we can expect it. If there are no surface-active agents, then at the moment we switch on the polarograph the mercury electrode is polarized completely because there is no electricity flowing. The voltage increases because the instrument increases the voltage at a constant rate, but the current doesn't increase. There is no electricity flowing, so we get a linear curve similar to the ionization constant of sugar and water. We get no oxygen maximum because there was nothing to conduct electricity to form this oxygen maximum. On the other hand, when we have impurities, although they are surface-active agent, they absorb the oxygen. At the same time, they would also permit a flow of electricity which would allow this oxygen maximum to occur. And thus we have a relation between the surface-active agents and oxygen which would allow the current to flow. The ionization indicates to us whether a sugar would floc or not.

If the ratio of abscissa distance divided by ordinate distance is higher than a certain value, it would be floc-free. If less, we would have floc-positive material.

J. Dowling: Do you aerate the samples at all?

Y. Yankelevits: On one occasion we tried aerating, but there was no substantial change in the polarogram.

J. Dowling: Do you know the drop mass of the mercury? This affects the drop time.

Y. Yankelevits: We use the standard capillary offered by the Sargeant Company. There are two types. One is for drop times between 2 and 5 seconds and the other is for drop times between 6 and 12 seconds. By cutting the capillary and reducing it in length, you would reduce your drop time to a desired level. We used the one between 6 and 12 seconds and cut it about one-third down. We use a constant drop time which is 4.5 seconds, and we can adjust it by simply adjusting the mercury reservoir at the top of the capillary. The mass of the mercury drop I cannot tell you, because I don't know the diameter of the capillary. We couldn't obtain this from the Sargeant people.

W.W. Blankenbach (B.C.): Was this work done on cane sugar or was any beet sugar used?

Y. Yankelevits: Entirely on cane sugar.

W.W. Blankenbach: So the validity of the polarograph in regard to beet sugar has not been established?

Y. Yankelevits: You are right, that has not yet been established.

REVIEW OF COLOR FORMING REACTIONS

by
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Introduction

The principal carbohydrate color substances formed during the production of raw sugar are melanoidins and caramels. The melanoidins contain nitrogen; caramels are nitrogen-free. This paper is concerned with the chemistry in the raw sugar mill related to melanoidin formation, namely the browning reaction.

From the outset of the processing of raw cane juice to the production of final molasses, conditions in the mill are conducive in some degree to the formation of melanoidins or browning polymers. These conditions are summarized in table 1. Some probable sugar chemistry related to color production during the clarification, boiling, and reboiling of sugar liquors will be considered in the light of these processes.

Probable Color-related Reactions Involving Sugars During Clarification

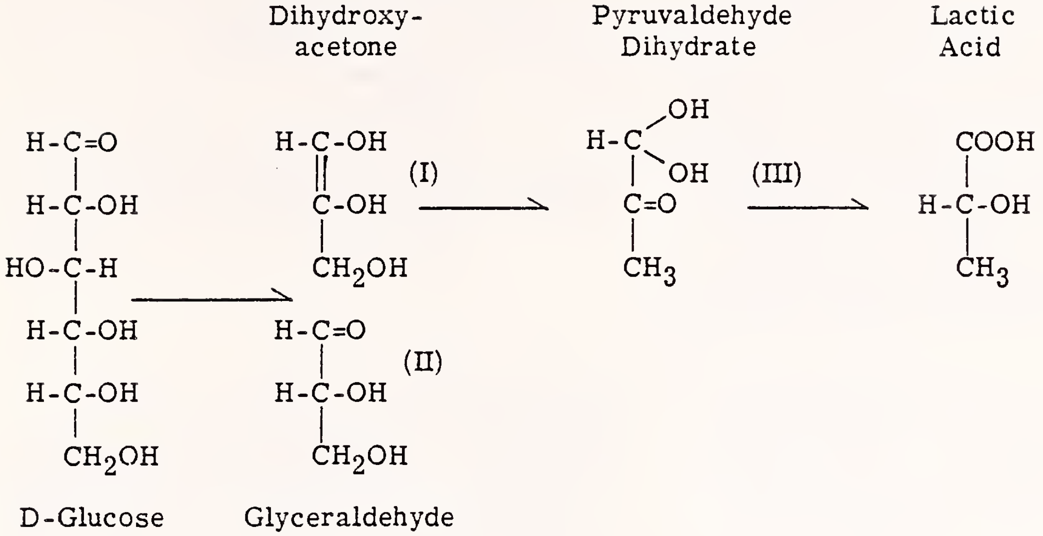
Some significant reactions occurring during clarification are fragmentations, condensations, rearrangements, and polymerizations. The principal juice-constituent hexoses, D-glucose, and D-fructose, are more susceptible to fragmentation than sucrose during this process. One probable course is shown in Reaction Series No. 1.

Three significant products are dihydroxyacetone (I), glyceraldehyde (II), and pyruvaldehyde (III) which can condense with other carbonyl compounds (aldol condensation) or with certain nitrogen-bearing substances as shown in Reaction Series No. 2-A.

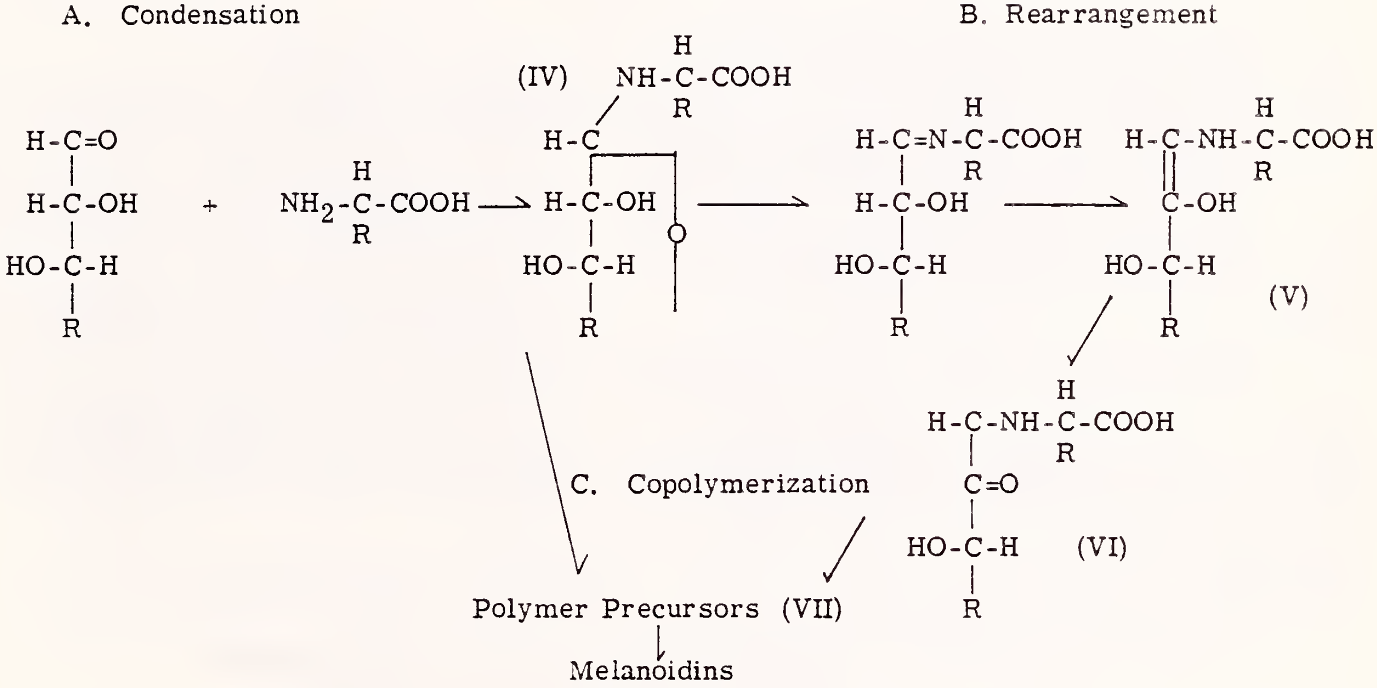
Table 1. --Some process environments in the production of raw cane sugar

Process environment	pH Shift	Temperature range, °F.	Duration of heating	Final solids content, (pct.)
Clarification	8.0 to 7.3	218 to 202	5 hrs.	12 to 18
Evaporation	7.3 to 6.5	230 to 150	30 min.	ca. 60
Boiling	6.5 to 5.2	- - -	- - -	80 to 85

Reaction Series No. 1. --Fragmentation



Reaction Series No. 2

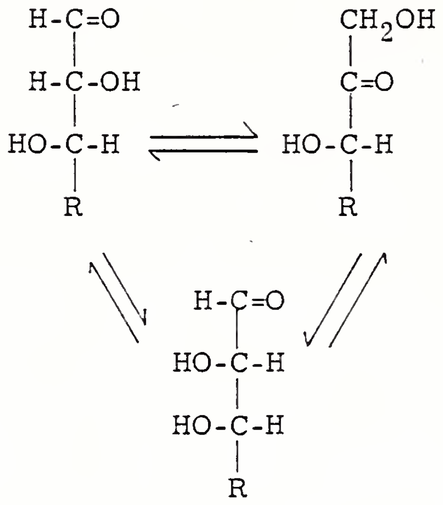


The simple nitrogen-bearing substances are glycosylamino acids (IV) which may condense yielding melanoidins precursors, (VII, course unknown) or engage in rearrangement according to Reaction Series No. 2-B (1) to form the highly reactive structure in V which can change readily to another glycosylamino acid (VI) and react further to become melanoidin precursors. Hexose interchanges occur during clarification and are attributed to the Lobry de Bruyn and Alberda Van Ekenstein rearrangement (2), as shown in Reaction Series No. 3. Hydroxyl and hydrogen ions promote these reactions. Carbon-14 tracer studies (3) have indicated that glucose-fructose interchange is extensive over the pH range from clarification to the final molasses. Mannose and psicose

Reaction Series No. 3

Lobry de Bruyn - Alberda van Ekenstein Rearrangement

D-Glucose



D-Fructose

D-Mannose

formation can be demonstrated by isotype studies (4). The major portion of the mannose is probably produced in the evaporated syrups of higher concentration. Some melanoidins are formed during clarification (5); however, the most significant production of these color bodies would appear to take place during the boiling and reboiling processes.

Probable Color-related Reactions Involving Sugars During Boiling and Reboiling

All of the significant sugar reactions involved in raw juice clarification occur also during the boiling and reboiling processes. In the latter environment these reactions take place with greater intensity due to the higher concentration of reactants. Reduced distribution in viscous liquors of heat from steam pipes is conducive to caramel formation (6). The accelerated browning reaction can cause a localized depletion of substances providing an environment favorable to caramel formation and to copolymerization. While the evidence for copolymerization is indirect, it is noteworthy. Glucose and alanine react to form a browning polymer in which the ratio of six-carbon residues to alanine residues is one to one (table 2). The

Table 2.--The ratios of six-carbon residues to nitrogen residues in certain browning polymers

Reactants	Repeating unit	Residues in repeating unit
Clarified limed cane juice	$C_{16-17}H_{21-22}O_{10}N$	2, 6 Carbon 1, 5 Carbon (amino acid)
Glucose + alanine	$C_9H_{10}O_{3-4}N$	1, 6 Carbon 1, 3 Carbon (alanine)
Glucose + alanine + sucrose	$C_{15-16}H_{20}O_9N$	2, 6 Carbon 1, 3 Carbon (alanine)

corresponding ratio in the polymer obtained from clarified cane juice is 2/1. The introduction of sucrose into the glucose-alanine reaction mixture affords a polymer with the same ratio between six-carbon residues and amino acid moieties as that obtained from cane juice. Sucrose appears to have either acted as a hexose donor or promoted copolymerization as shown in Reaction Series 2-C

Summary

Probable reactions involving sugars and leading to the formation of colored substances during the production of raw cane sugar are fragmentations, condensations, rearrangements, polymerizations, and copolymerizations. These reactions are intensified in the processing of syrups of higher concentration with the probable formation of caramels and increased yields of melanoidins.

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DISCUSSION

J. Dowling (Refined Syrups & Sugars): There was a lot of organic chemistry in this paper and a lot of compounds and I am wondering if they were all identified truly by Nuclear Magnetic Resonance (NMR) or by other means, or is this just theory?

W.W. Binkley (N.Y. Sugar Trade Lab.): As far as NMR is concerned it is valuable as supporting evidence for everything that we know about these reactions. All the conclusions derived from work in our laboratory were based on Carbon 14 radiotracer chemistry. Results obtained by this approach are reliable; caution should be exercised in the use of IR (Infrared), or NMR data. For example, the basic work on the chair configuration was done at Louisiana State University by Professor Reeves; his findings are held in high regard. The role of conformation and bond positions in these reactions is still in the wain-and-see stage.

J. Dowling: I think the chair form is very stable, but the boat form, has for years been argued against. Yet you state that the basic configuration is in the boat form, which is against principle as far as stability goes.

W. W. Binkley: Let me try to clarify this—since the fructose moeity in sucrose is in the furanose form which is a five-member ring, not six, it can assume the envelope conformation readily but not that of a boat. NMR spectra also favors the envelope conformation over that of the skeletol atoms all being in the same plane. You are correct in your statement that the chair form is the stable configuration for pyranose sugars. The boat form is rarely encountered.

S. Borodkin (American): Do these color reactions involve only traces of carbohydrates or is there a pretty large amount of carbohydrate involved in the colorant forming process?

W. W. Binkley: First I would like to say that I am unfamiliar with what takes place in commercial operations. Polymerization and copolymerization in the laboratory, with a model experiment, require considerable amounts of reactants to obtain small amounts of polymer products. We use cane juice (usually lyophyillized cane juice) as our starting material.

G. A. Hageney (American): Do you know the relative importance of each of these reactions? In other words, how much color do you get from the rearrangement, fragmentation, copolymerization?

W. W. Binkley: I wish I did. I have some opinions on it though if you want to hear an opinion. First, I think that in clarification—the browning reaction, the condensation between the amino acids or the amine compounds and the hexoses, like glucose, fructose or mannose, is the dominant reaction. Some browning polymers are formed also. In the boiling and reboiling, the browning reaction is accelerated. Conditions are more favorable for caramel formation and copolymerization, in these processes. The shift in pH and the increase in the concentration of the reactants from clarification to reboiling favor these reactions.

W. A. Clarno (Savannah): There are innumerable combinations, probably of this browning reaction in different amino acids and the different sugars. Is there a generalization that can be made on critical temperatures at which these reactions occur? Once you have reactions like these starting you have probably many of them going on in the same piece of equipment. Some of the reactions are exothermic, therefore, there must be maximum temperatures that cannot be exceeded.

W. W. Binkley: The present state of our knowledge does not provide an answer. The results have been known to you people for a long time. Now we are trying to explain what takes place. If the pH is high, certain unfavorable fragmentations will occur. Thus high pH should be avoided. On the other hand low pH and high temperatures will also degrade sugars. High temperatures are at present unavoidable. The problem of low pH is less serious, a blackstrap very seldom is below pH 5. We have had molasses in our Laboratory that have been overheated and in this instance extensive polymerization had occurred.

W. A. Clarno: We have had experiences with raw sugar, for instance, that has been shipped under too high temperature conditions where the pH of the raw sugar has gone down to 4.0 and 4.1. This has ended in a very drastic browning reaction.

W. W. Binkley: Yes, somewhere in all of these reactions must be optimum temperatures for the commercial processes. I do not believe such data have been published. It is an important area for research and work should be started. If moisture accumulates on the surface of raw sugar crystals, reaching 15 to 20 percent in localized areas, the browning reaction proceeds rapidly. This reaction is accelerated at temperatures in the neighborhood of 100° C.

L. F. Martin (SURDD): There is this one observation I can make on this question that Clarno has raised. I don't know what you can do about it because you do work, and must work at high concentrations. As far as the reactivity of the sugars is concerned, the concentration is the big factor. We have been studying the rearrangement to mannose and it does not take place at the concentration of the juice. We have tried to find it with chromatography and electrophoreses, but it is not there. However, it does appear in the sugar boiling, and it is in the molasses. So apparently at low concentrations you can heat at the temperature of the clarifier, which is higher than in the vacuum pan, and no reaction occurs. Whereas at high concentration, even at the lower temperatures of the pan you get quite a bit of mannose.

THE pH SENSITIVITY OF SUGAR COLORS AND EASE OF DECOLORIZATION

by
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Sensitivity of the attenuation index of washed raw sugar liquors to pH changes shows a positive correlation with ease of decolorization in laboratory bone char columns.

Changes in the pH sensitivity are followed through laboratory processing of raw sugar samples. Processing steps discussed are affination, defecation with phosphoric acid and lime, decolorization by bone char, and crystallization.

Colored sugars prepared by caramelization, alkaline degradation, or melanoidin formation are found to have pH sensitivities lower than those shown by raw sugars. Absorption spectra of these products, measured at various pH values, are presented.

Introduction

Raw sugar varies widely in color as well as in the ease of separation of the colorants by decolorization with bone char or by crystallization. These are familiar problems of the refiner. By examining the properties of sugar color we may learn how to cope with these problems more effectively. We have applied the sensitivity of sugar color to pH changes as a criterion of the variation in composition of the colorants found in raws processed at the Crockett refinery. This report presents our findings using this approach.

Most of us are aware of the more familiar color forming reactions. In introducing this paper, I would like to refer to examples from the literature which relate specifically to the pH sensitivity of sugar colorants.

McLaren (1) isolated a canary yellow substance from molasses. Alkali intensified the color of this material which he believed to be composed of free flavanoids and flavanoid glucosides extracted from the cane. The color of another fraction isolated by McLaren, the polyphenols, depended even more on pH (2).

A characterization of synthetic colorants was made by Persson (3). He showed that the color of melanoidin was less sensitive than that of caramel to pH changes. Further, the caramel colorant was removed by carbon more efficiently than was melanoidin.

Tu attributed pH sensitivity to the thermal degradation products of reducing sugars (4). Ahari (5) prepared colorants by caramelization of sucrose, alkaline degradation of glucose, and reaction of amino acids with glucose to produce melanoidins. He showed that the color of melanoidin was less pH sensitive than the other two. Caramel and alkaline degraded glucose colors increased about 70 percent between pH 4 and pH 9. In addition, Ahari found that melanoidin had the smallest tendency to produce dark sugars by crystallization, and caramel the greatest--the percentage of color entering the crystal was three times as high with the caramel as with the melanoidin.

Deitz (6) stated that fractionation of colorant in bone char column effluents was indicated by a change in pH dependency from that of the original liquor. The color of the first effluent had virtually no pH sensitivity, and the sensitivity of subsequent effluent approached that of the on-liquor.

It was suggested by Carpenter and Deitz (7) that the "indicator effect," as the effect of pH on color may be called, may be useful to measure a class of impurities, since some sugars show a greater pH effect than others. As they pointed out, however, the absolute amount cannot be determined by transmission measurements alone because turbidity affects pH sensitivity.

The data presented here were obtained in examining the indicator effect. To measure this we determined the attenuation of liquors at pH 9 and at pH 4. The ratio of these colors, i. e., that at pH 9 to that at pH 4, is a measure of pH sensitivity. For convenience we refer to this ratio as the "indicator value" or I. V. In addition to looking at the relationship between pH sensitivity and decolorization in a large number of washed raw sugars, changes in pH sensitivity which occur during different stages of processing were followed.

Alakline degradation products and melanoidins are generally considered to be major contributors to the color of raw sugar. Because attenuation measurements over a wide range of wavelengths can provide a better characterization of the properties of the colorants than measurements at a single wavelength, the spectra of these substances were compared with those of washed raw sugar. The comparison includes changes in spectra caused by varying pH.

Treatment of Samples

Samples of whole raw sugars were collected in the refinery at intervals. After laboratory mingling with a refined sugar liquor and centrifugation, the samples were air-dried, bagged, and stored for later use. Liquor samples were prepared from these washed sugar samples by liming a 66.5° Brix solution to pH 7.2 at 80° C, addition of 1.5 percent Filter Cel on solids, and vacuum filtration. If defecated, phosphoric acid (0.006 percent P_2O_5 on solids) was added prior to liming. Decolorization tests were made using laboratory char columns and auxiliary equipment described by N. L. Pennington (8) at the 5th Bone Char Technical Session.

Color Measurements

For convenience, the term "color" used here refers to 1,000 times the attenuation index. Unless otherwise specified, this value was determined at 420μ and pH 7. Measurements at pH 4 and pH 7 posed no special problems. When samples were prepared for measurement at pH 9, the pH was found to drift to lower values with time. Use of a buffer was necessary to maintain the higher alkalinity.

Liquor Colors and pH Sensitivity

The range of liquor colors from a large sampling of washed raws, before and after char decolorization, without phosphoric acid defecation, is shown in Figure 1. The expected relationship--darker on-liquors tend to produce darker effluents--is evident. It can also be shown that lighter on-liquors decolorize slightly more efficiently. For example, an on-liquor with a color of 500 produces an effluent with a color of about 35, or 7 percent remaining. But an on-liquor twice as dark yields an effluent with a color of about 80, or 8 percent remaining. This effe is expected if adsorption satisfies an equatio like the one proposed by Loebenstein (9) to describe column decolorization. But Figure illustrates two problems related to the subject of this paper. First, what is the relatio ship between the on-liquor color, which vari from less than 500 to over 1,000, and the pH sensitivity of the color? Second, the spread in effluent color for a given on-liquor color i beyond the experimental errors of determination. Is this wide variation in ease of decolo ization related to pH sensitivity?

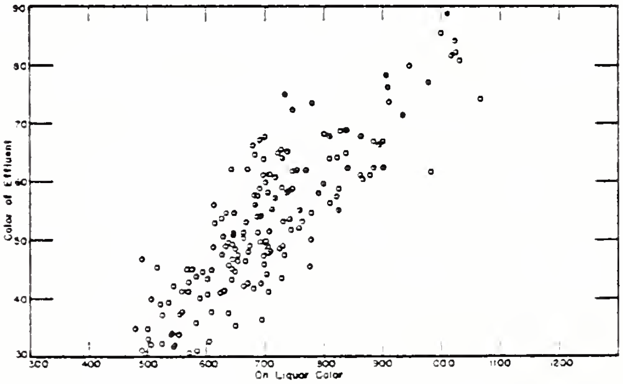

Figure 1.--Variation in color of bone char decolorized washed raws with color of on-liquor (1000 X a^*_{420} at pH 7.0).

The first relationship is illustrated by Figure 2. The ratio (attenuation index at pH 9/attenuation index at pH 4) or I. V., is plotte on the Y-axis of Figure 2. Along the X-axis are plotted the pH 7 colors of the liquors. Th points follow an inverse relationship. That i the smaller values of I. V. are found in the darker liquors, while the more pH sensitive colors are found in the lighter colored liquors

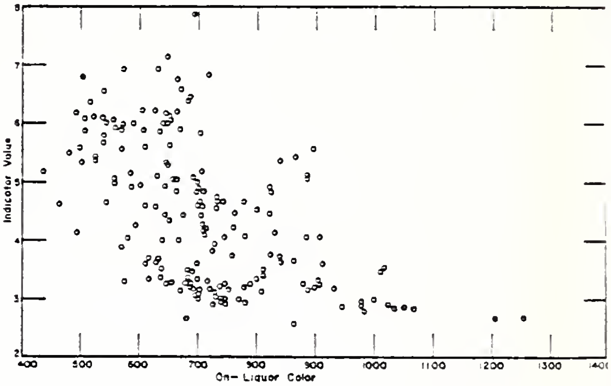

Figure 2.--Variation in sensitivity of color to pH changes (ratio of pH 9 color to pH 4 color) with color of on-liquor (1000 X a^*_{420} at pH 7.

The second relationship is presented in terms of the variation in the percentage of color remaining after bone char decolorization, plotted against the I. V. of the on-liquor This is shown in Figure 3. Although the points are well scattered, a relationship is evident. Poor decolorization, shown by the high percentage of color remaining after bone char decolorization is associated with a low I. V.

The refiner is concerned with dark-colored effluents whether they come from

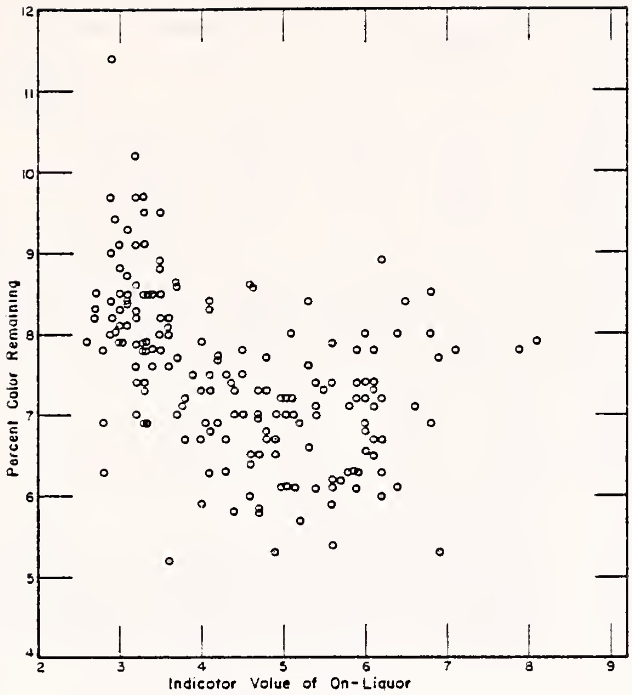

Figure 3.--Variation in ease of decolorization (average percent color remaining in bone char column effluent) with indicator value of on-liquor.

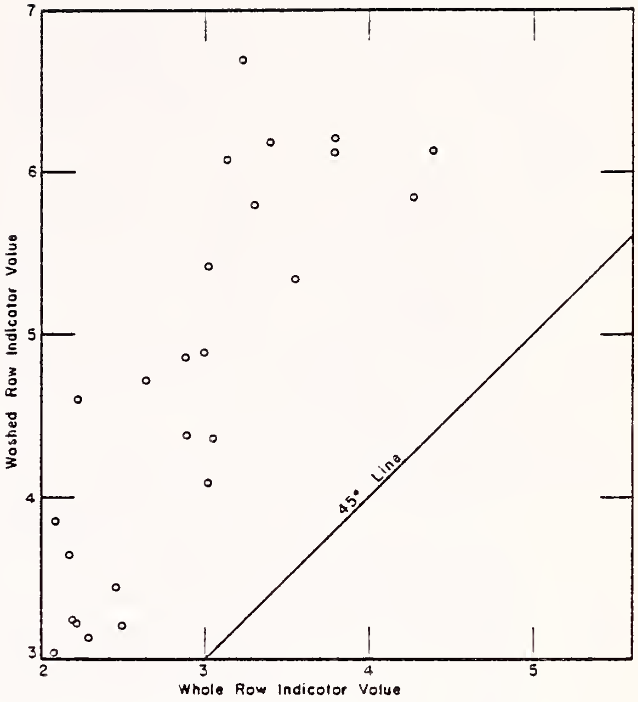

Figure 4.--Effect of laboratory affination on the sensitivity of sugar color to pH changes.

exceptionally dark raws or from light raws which decolorize poorly. It appears that both of these problems may be related to a low pH sensitivity of the color in these raws.

Processing and pH Sensitivity

Affination. --To determine the pH sensitivity of whole raw sugar color, a number of whole raw samples were dissolved to give about 10° Brix solutions, and vacuum filtered with Filter Cel to remove colloidal matter. Colors were measured on portions of the filtrate after adjustment to the appropriate pH values. Washed raw liquors from the same whole raws were prepared and colors determined as described earlier. The calculated values of I. V. for washed raws were plotted in Figure 4 against those of whole raw sugars. Points of no change in I. V. would fall on the 45° line. The I. V. of these samples was increased 30 to 100 percent by affination--the average increase was about 60 percent--from an average I. V. of 1. 9 to an average of 3. 1.

When we measure sugar color at pH 4, the effect of pH sensitive colorant on attenuation is minimized. At pH 9, the effect of pH sensitive colorants on attenuation is emphasized. An increase in indicator value represents an increase in the proportion of total colorant that is pH sensitive.

The increase in I. V. of sugar resulting from removal of the molasses film suggests the possibility that in crystallization of raw sugar, the pH sensitive colorant tends to be incorporated in the crystal. We will come back to this point later.

The relation of affination to pH sensitivity can be shown in a manner more meaningful to the refiner. We can measure, at different pH levels, the amount of color in raw sugar before and after affination. The data representing several raws are presented in table 1. For these samples, the average I. V. was increased from 2. 3 to 3. 6 by removing most of the molasses film surrounding the crystal. In terms of efficiency of total color removal, however, we can see that the percentage of color remaining in the washed raw sugar increased with the pH of measurement. For the average sugar in table 1, 18 percent of the color remained when relatively pH insensitive color was measured (i. e., at pH 4. 0). When the effect of pH sensitivity was emphasized by making the color

Table 1.--Removal of color by affination: Effect of varied pH of measurement

	pH of Measurement	Sample No.					Average	
		1	2	3	4	5		
Whole raw color (1000 a^*_{420})	4	2100	3010	2530	4200	3350	3040	I. V. = 2.3
	7	2980	3910	3650	5620	4780	4190	
	9	5160	6720	6670	8760	7530	6970	
Washed raw color (1000 a^*_{420})	4	435	465	505	640	685	545	I. V. = 3.6
	7	775	710	845	910	1100	870	
	9	1580	1800	2240	2220	1960	1960	
Percent color remaining after affination	4	20.7	15.4	20.0	15.3	20.4	18	
	7	26.0	18.2	23.1	16.2	20.9	21	
	9	30.6	26.8	33.6	25.3	26.1	28	

measurement at pH 9, 28 percent of the color was left in the washed sugar. To the extent to which raw sugars show an indicator effect, color cannot be a quantitative measure of the amount of colorant or other impurities in the sugar, even when color is measured at a specified pH.

Defecation. --An increase in I. V. accompanies the removal of colorant and other impurities by defecation with phosphoric acid and lime. Figure 5 illustrates this. The I. V. of defecated washed raws were plotted against the corresponding values for the washed raws filtered without defecation. As in the previous figure, the points lie above the 45° line. The increase in I. V. averaged about 10 percent.

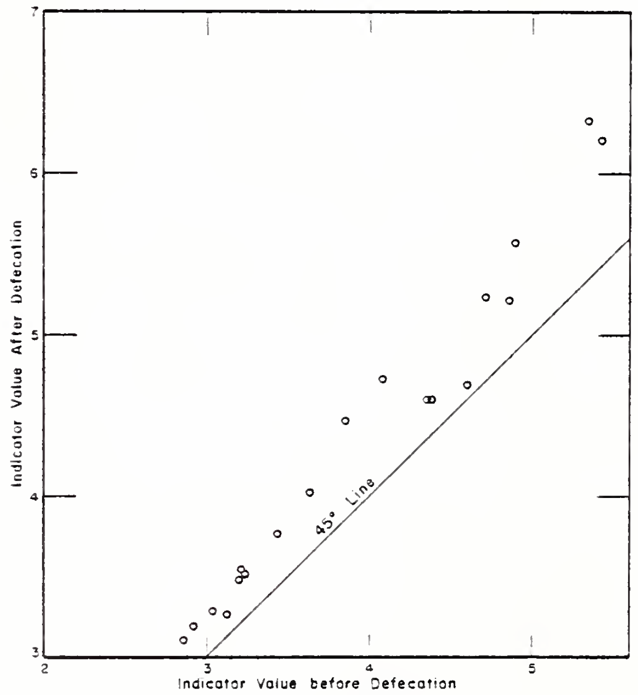

Figure 5.--Effect of phosphoric acid defecation on the sensitivity of color to pH changes.

Because defecation and filtration involve the removal of suspended matter, we should consider the relationship between I. V. and turbidity. How much of the variation in pH sensitivity is caused by differences in the colloidal content of the liquors tested? The relative turbidities of a set of well filtered washed raw sugar liquors were determined by measuring the amount of light scattered at right angles to the incident beam, using an Aminco Absolute Light Scattering Photometer. The ratio of the intensities of scattered to transmitted light is a measure of the amount of colloidal matter in the liquor. It has been found that pH has virtually no effect on the attenuation due to turbidity (7). That is, turbidity will contribute to the apparent color in the same manner as pH insensitive colorant. Thus, highly turbid liquors will tend to have low I. V.'s.

In Figure 6, curve A represents the relative turbidities of the set of samples tested, plotted on a logarithmic scale against I. V. We see that liquors of higher I. V. tend to have lower turbidities. Is the variation in turbidity sufficient to account for the wide range in I. V. observed? To answer this question, dissolved washed raw sugars were filtered with several grades of filter aid. The turbidity and I. V. of each sample were determined. Curve B of Figure 6 shows the

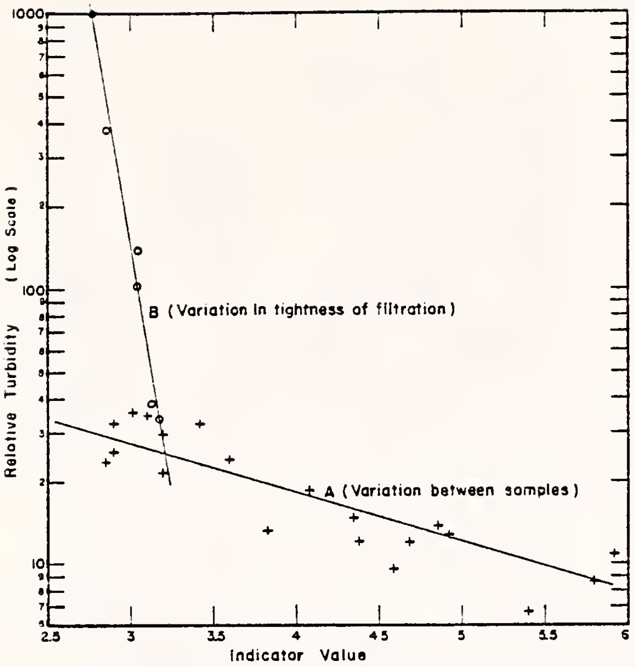

Figure 6.--Variation in turbidity with sensitivity of color to pH changes.
Curve A: For different samples of washed raw sugar liquors filtered with equal tightness.
Curve B: For a single liquor when tightness of filtration is varied.

results for a typical washed raw sugar: A large decrease in turbidity produced only a small increase in I. V. This effect can account, at least in part, for the change in I. V. by defecation, but is too small to account for the variation in I. V. between different raws. The relationship shown in Curve A of Figure 6 has not been accounted for. We can speculate that the contribution of pH insensitive colorant to turbidity may be greater than that of pH sensitive colorant because of a larger molecular size.

Bone Char Decolorization. --We have seen how the first stages of refining produce an increase in the sensitivity of sugar color to pH changes. And data were presented in Figure 3 which suggest a correlation between a high I. V. and good bone char decolorization. Because of this correlation we should expect to find that bone char preferentially adsorbs pH sensitive colorant. In fact, the previously cited report of Deitz (5) supports this view. He found that the first effluent off of char contained colorant not affected at all by changes in pH. The data presented here can add to the picture. Laboratory bone char column decolorization tests were made on the group of washed raws previously tested. The data are presented in Figure 7, where the effluent I. V. after one and three displacements is plotted against the I. V. of the on-liquor. The data show that the I. V. of the char column effluent increased with the I. V. of the on-liquor. For samples of low I. V., the results were consistent with those obtained by Deitz. That is, the I. V. of the effluent was lower than that of the on-liquor and increased toward that of the on-liquor as more liquor was displaced through the column. However, for on-liquors of high I. V., the I. V. of the effluent, while lower at the start, was higher after three displacements than that of the on-liquor. This effect is indicated by points above the 45° line of Figure 7.

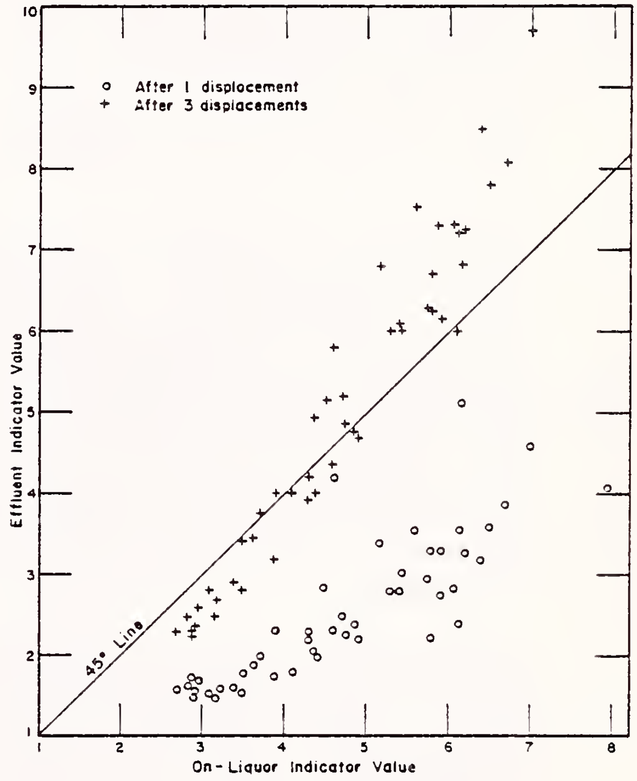

Figure 7.--Effect of bone char decolorization on the sensitivity of color to pH changes.

The relatively poor bone char decolorization of liquors of low I. V. was discussed earlier. A partial explanation for differences in decolorization based on the indicator effect can now be considered. Although lighter in color at pH 7, liquors of high I. V. may contain more pH sensitive color than liquors of low I. V. This is illustrated in Figure 8, in which the colors of two liquors, differing greatly in

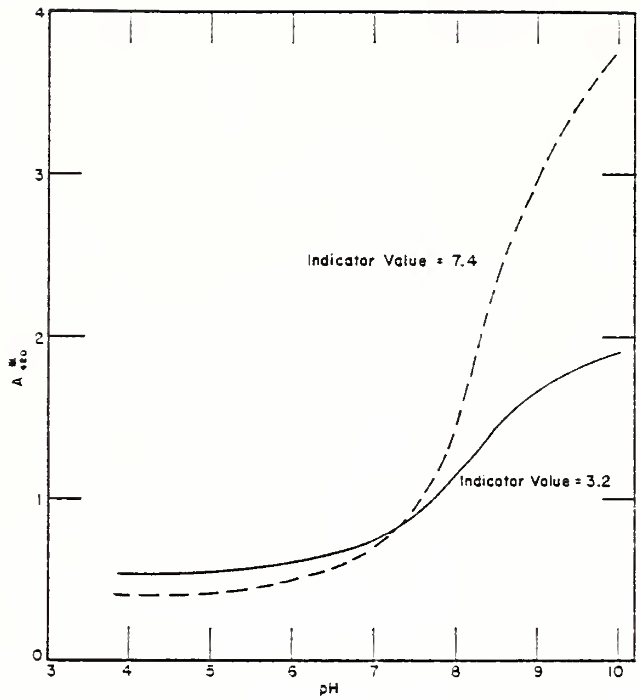

Figure 8.--Effect of pH on color of washed raws with high and low indicator value.

I. V., are plotted against pH. Since color measured at pH 4 is darkest for the liquor of low sensitivity, we can assume that this sample contains more of the pH insensitive color. However, the liquor which is lightest when measured at pH 4 (and has a high I. V.) is the darkest pH 9 liquor. If this sample contains less pH insensitive color than the other sample, it must contain more pH sensitive color.

Bone char in a new column fractionates sugar colorants by selectively adsorbing pH sensitive colorant from the liquor. Hypothetical curves representing relative rates of adsorption of pH sensitive and insensitive colorant by bone char are shown in Figure 9. For a liquor containing a high proportion of pH sensitive colorants the rate of adsorption of these colorants is initially much higher than that of pH insensitive colorant, as illustrated in Figure 9A. As decolorization progresses, the rate of removal of this colorant decreases faster than the rate of removal of pH insensitive colorant. This produces effluent of increasing I. V. If the rate curves cross, effluent of higher pH sensitivity than the on-liquor will be produced. The char will then be selectively adsorbing pH insensitive colorant. The variation in I. V. in the example where the rate curves cross is shown in Figure 9B. Consider, now, an on-liquor of low I. V. In comparison with the above example the initial rate of adsorption of insensitive colorant will be higher and that of sensitive colorant lower because the liquor contains more pH insensitive colorant and and less pH sensitive colorant. But because of differences in the extent to which the char is loaded with the two types of colorant, the rate of removal of pH sensitive colorant could remain higher than the rate of removal of pH insensitive colorant at all stages of decolorization (Figure 9C). The effluent would then maintain a lower I. V. than shown by the on-liquor (Figure 9D).

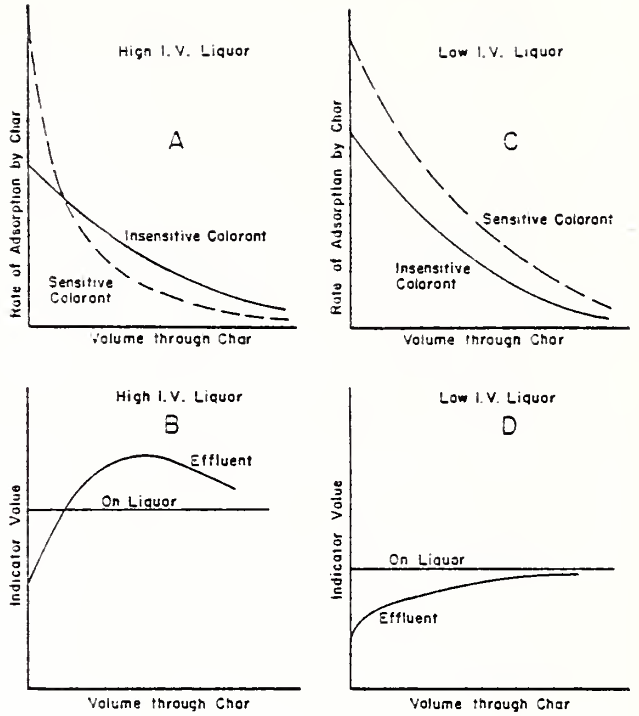

Figure 9.--Hypothetical curves for rates of adsorption of pH sensitive and insensitive colorant by bone char, and I.V. of effluent, plotted against volume through char.

This presentation oversimplifies the situation. We have neglected the possibility that the color which appears when pH is increased represents more than one pH sensitive colorant. Supporting evidence is shown in Figure 10. The percent color remaining after char decolorization in a typical test is plotted against volume through char. As previously shown in the early stage of decolorization pH sensitive colorant is preferentially adsorbed. We find that the percent color remaining in the

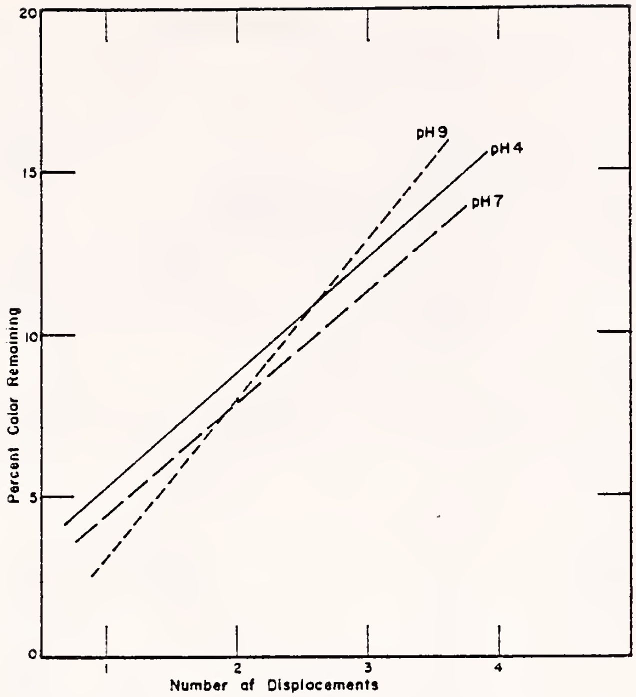

Figure 10.--Effect of pH of color measurement on the percent color remaining in a sugar liquor after decolorization in a laboratory char column.

first effleunt is lower for color measured at pH 9 (representing primarily pH sensitive colorant) than for color measured at pH 4 or 7. In the latter effluent, pH 9 color shows the highest percent color remaining or the poorest decolorization. In contrast, however, the pH 4 and 7 curves are parallel. That part of the total pH sensitive colorant that produces color on going from pH 4 to pH 7 is adsorbed better than the insensitive colorant at all stages of the test. This is shown by the lower percent color remaining when pH 7 measurements rather than pH 4 measurements are used to calculate the decolorization efficiencies. Since this is not true for the increase in color from pH 7 to pH 9, these results indicate that there is more than one type of pH sensitive colorant.

Crystallization.--Recently the study of pH sensitivity was extended to include crystallization. Thus far only a few tests have been made. For this purpose a small glass apparatus with provisions for heat and vacuum control, agitation, seeding, and continuous feed was used. The centrifuged sugars from the experimental strikes were alcohol washed to remove the syrup film. Color readings on solutions of these sugars thus represent only the substances contained within the crystal.

In Figure 11, the I. V. of the crystal and the ratio of crystal color to liquor color are each plotted against the I. V. of the bone char decolorized liquor before crystallization. The I. V. of the crystal increases with the I. V. of the liquor. Furthermore, since the points fall above the 45° line of Figure 11A, crystal I. V. is higher than the corresponding liquor I. V. This is evidence that pH sensitive colorant tends to be preferentially included with the crystal and is consistent with the conclusions of the study of the effect of affination on changes in I. V. On this basis, we would expect the ratio of crystal to liquor color to be higher for samples of higher I. V. Except for a few high ratios for some samples of low I. V., this is suggested by the data as shown in Figure 11B. Generally, the high I. V. samples did not produce sugars of as low a crystal to liquor color ratio as did the low I. V. samples.

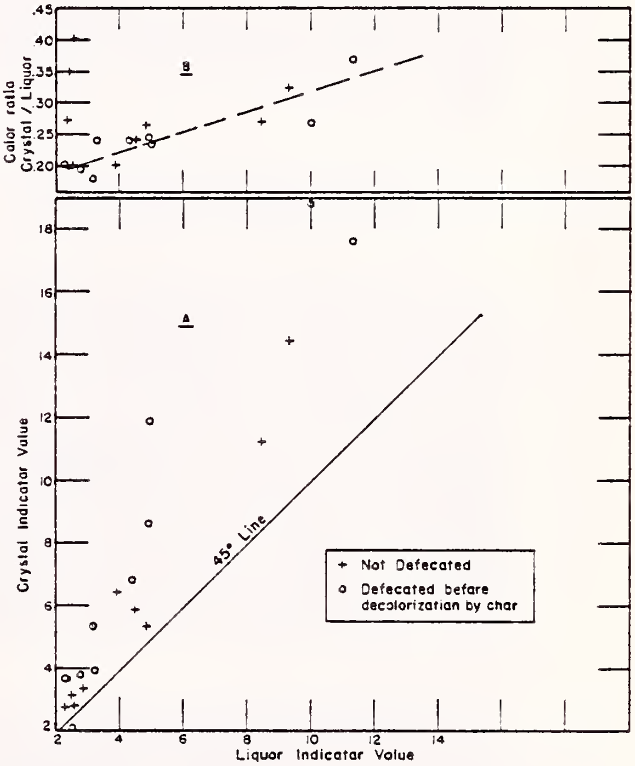

Figure 11.--(A) Indicator value of the crystal and (B) ratio of crystal to liquor color plotted against indicator value of bone char decolorized liquor before crystallization.

In the refinery, the char effluents sent to the pans do not have as high I. V.'s as those found in the laboratory tests. This is due in part to the mixing of char effluents, in the refinery, from filters of varying age. In addition, the liquors crystallized in the experimental pan were the fifth and sixth displacements from laboratory char columns (earlier effluents were used for color and other determinations). Thus, the liquors crystallized in the laboratory contained proportions of pH sensitive colorant higher than typical refinery liquors.

These factors explain why the ratios of crystal color to liquor color in Figure 11 exceed a value of 0.20, which is about twice that found in normal refinery operation. Nevertheless, I.V. may find value in predicting ease of decolorization through crystallization, as well as through bone char treatment, when raws of variable quality are refined. As we have shown, raws that are light in color tend to have a high I.V. and, therefore, produce exceptionally well decolorized liquors by bone char column decolorization. By the same token, because high I.V. liquors tend to be light in color, the inefficiency in color removal from these liquors by crystallization is offset to a considerable degree by their lower color level.

Measurement of Absorbency of Light at Different Wavelengths

The discussion of pH sensitivity thus far has involved measurements at only 420 mμ. Measurements at this wavelength, which is within the visible range of 400 to 700 mμ, supply information useful to the refiner, concerning the effect of colorants on the visual appearance of sugar. By extending our measurements to a wavelength range covering much of the visual and near-ultraviolet, we may obtain information concerning the chemical nature of the colorants. Spectra were measured with a Beckman model DU spectrophotometer.

The spectrum of a typical washed raw sugar liquor from 220 to 500 mμ, is presented in Figure 12. Spectral measurements were made on samples adjusted to pH 4, 7, and 9, before and after bone char column decolorization. Several features of the spectra are pertinent to our discussion.

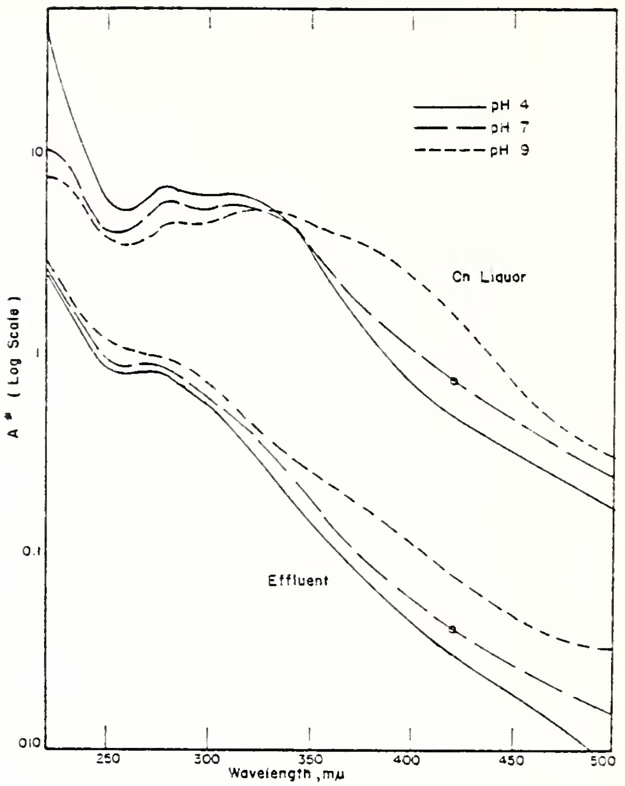

Figure 12.--Spectra of washed raw sugar liquor before and after decolorization by bone char, determined for liquors at pH 4, 7, and 9.

Throughout the visible range, absorption of light by the on-liquor, represented by the upper set of curves, increases with pH. The pH sensitivity is greatest at about 380 mμ. The absorption at pH 9 is over three times that at pH 4. Our standard of 420 mμ is indicated by the small circle in Figure 12. At about 330 mμ, the absorption is relatively independent of pH, and below this wavelength, a reversal in sensitivity is seen. The magnitude of this reversal varied considerably in other samples. Absorption maxima in the range of 275 to 320 mμ are evident in Figure 12, but are less prominent in other samples.

The lower curves of Figure 12 are spectra of bone char decolorized liquor. Here we find that absorption increases with pH through the visible and near ultraviolet range. The pH sensitivity at 420 mμ has decreased to an I.V. of 2.5. This decrease in sensitivity resulting from bone char treatment was discussed earlier. Since the absorbency is plotted on a logarithmic scale, the arithmetic difference between the upper and lower curves measures absorbency ratios or the efficiency of decolorization. The difference is greater in the visible region of the spectrum than in the ultraviolet. For example, the difference in absorbency for the pH 7 solution at 420 mμ indicates about 6 percent color remaining; at 280 mμ the corresponding value is about 15 percent. This, fortunately for the refiner, shows that colorants are removed by char to a greater extent than colorless nonsugars which absorb light only of short wavelengths.

Examples of spectra of sugar solutions which contain colorants prepared in the laboratory are shown in Figure 13. The colorants were made by the caramelization of sucrose, the alkaline degradation of invert sugar, and the reaction of invert sugar with an amino-acid. The general shape of their spectral curves are similar and resemble that shown in Figure 12 for a washed raw sugar. The prominent absorption peaks at 230 and 284 mμ in the spectrum of caramelized sucrose indicate the presence of hydroxymethyl furfural (10). The formation of this chemical may account for much of the ultraviolet absorption shown in the other curves of Figure 13. However, only the colorant produced by alkaline degradation of invert shows a significant amount of pH sensitivity. Although several attempts were made to produce colorants of high sensitivity, a maximum I. V. of less than 2 was reached. In addition, the I. V. is not at a maximum in the range of 380 to 400 mμ, as it is with raw sugar color. These results suggest that the colorants responsible for the relatively high I. V. of raw sugar are not produced by the same reactions we have applied.

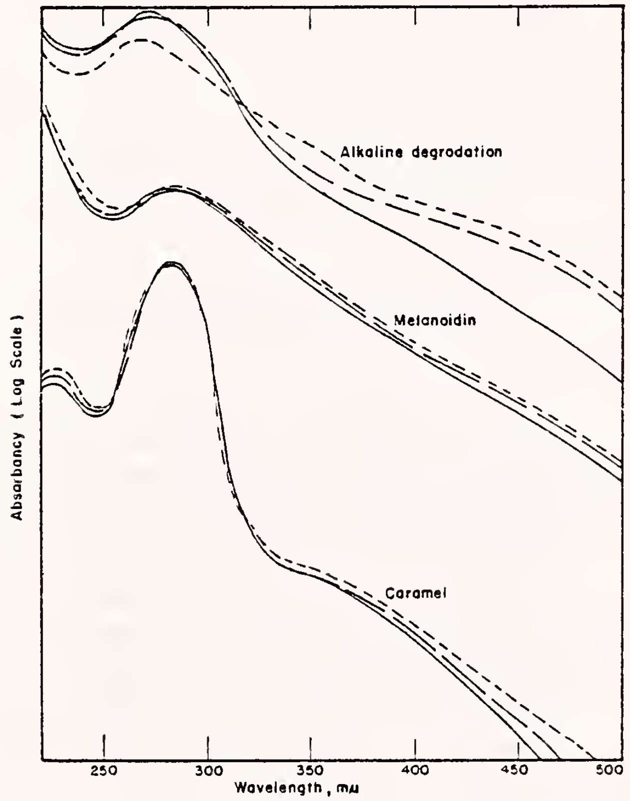

Figure 13.--Spectra resulting from reactions of sugars, determined at pH 4, 7, and 9.

The effect of heating washed raw sugar liquor under alkaline conditions were examined. The spectra of a liquor of initially high I. V., before and after heating at 65° Brix, pH 10, and 80° C. for 2 hours are shown in Figure 14. A significant increase in the pH 4 and 7 absorbencies, and a decrease in the pH 9 absorbency are apparent. (Colors measured at 420 mμ are designated in the figure for reference). These spectral changes are attributed to a conversion of pH sensitive to pH insensitive colorant. The conditions were

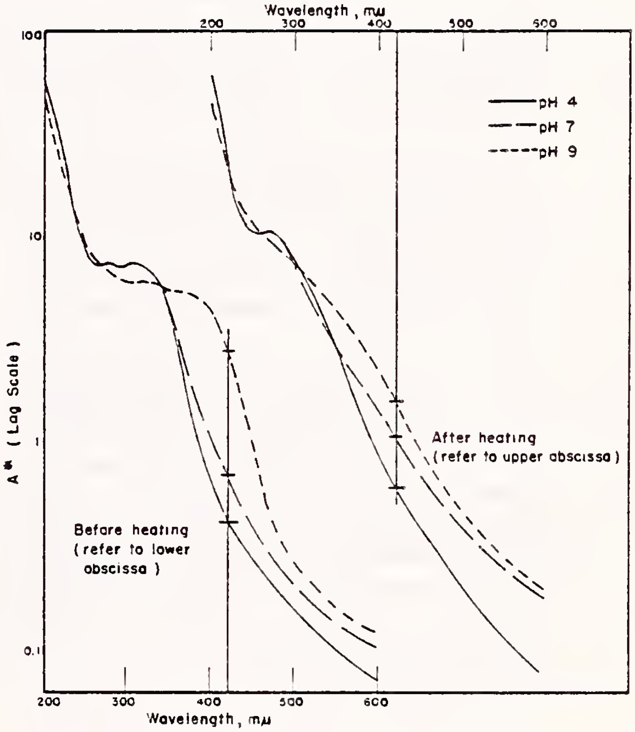

Figure 14.--Spectra of washed raw sugar liquor before and after heating at 66.5° Brix, pH 10, 80° C. for 2 hours, determined at pH 4, 7, and 9.

extreme, and it is unlikely that like conditions exist at any stage of factory processing. However, it is reasonable that other conditions which do exist during raw sugar manufacture could cause similar changes. The conversion of pH sensitive to pH insensitive colorant may be a basis for the correlation between high color and low I. V.

In a number of additional tests, it was found that when alkaline liquors of initially low I. V. were heated, absorbencies measured at pH 4 and 7 increased to a lesser extent than was shown by high I. V. liquors. Also, the pH 9 absorbency did not decrease. This further suggests that pH insensitive colorant is a modified form of the pH sensitive colorant--in the liquor of low I. V. the change has already occurred and insufficient pH sensitive colorant remains for significant additional conversion.

Since the color of raw sugars is generally doubled between pH 4 and 7, the attenuation index used by the refiner represents primarily colorant of which at least half is pH sensitive. Evidence has been submitted which suggests that this half is not derived simply from the degradation of sugar. One alternative is that some of the colorant may be polyphenols or their derivatives. This class includes naturally occurring colored pigments as well as colorless compounds which require alkaline conditions, enzymatic action, oxidation, or combination with iron salts to produce colorants (11). For example, it has been demonstrated that sugarcane tannin is derived from d-catechin, which is structurally related to the flavanone pigments that occur in most plants (12). Although catechin is colorless, its alkaline solution will darken on exposure to air. The spectrum of the colored solution, as shown in Figure 15, demonstrates a pronounced pH sensitivity of the colorant in the visible region.

Summary

A relationship has been found between the sensitivity of sugar color to pH changes and ease of decolorization. This relationship shows that the more sensitive the sugar color is to pH changes, i. e., the higher the I. V., then the easier it is to decolorize by bone char.

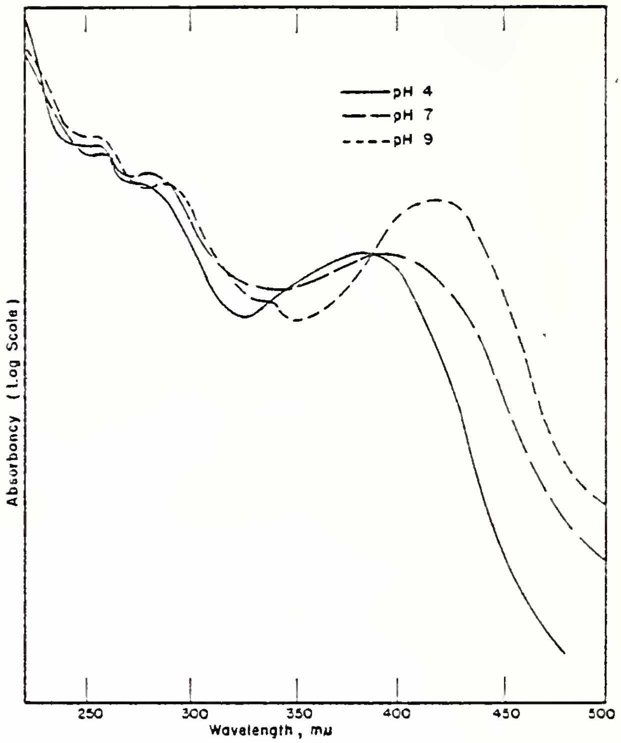

Figure 15.--Spectra of colorant produced in alkaline catechin solution, determined at pH 4, 7, and 9.

An increase in the I. V. of sugar is produced by affination, defecation, and crystallization. These increases are generally not a result of the removal of colloidal substances that contribute to turbidity but indicate a fractionation of colorant. Performances of washed raw sugar liquors in char column tests indicate that char adsorbs pH sensitive color at a greater initial rate than it adsorbs pH insensitive color. pH sensitive colorant tends to be included in the crystal during crystallization of bone char decolorized liquors.

The high indicator effect shown by washed raw sugars was not found in colorants produced in the laboratory. The colorant responsible for sensitivity apparently can be converted by suitable reaction conditions to a darker but Dirtless pH sensitive colorant. This change may account for part of the difference in color and sensitivity of color among sugars of varying refinability. It is suggested that a significant part of pH sensitive sugar colorant may be derived from naturally occurring polyphenols.

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DISCUSSION

W. North (Tate & Lyle): Could I ask Dr. Smith whether the raw sugars you refer to are primarily from the Hawaiin Islands, and secondly, have you examined the comparative effectiveness of other adsorbents than bone char on the removal of pH sensitive color?

N. H. Smith (C&H): We used Hawaiin raws. However, I have measured a few other samples that I had access to. They also varied in the pH sensitivity. They tended to be at the less pH sensitive end of the spectrum of sensitivities.

In regard to your other question, I did look at other adsorbents. Granular carbon seemed to have an even greater effect on the sensitivity of color to pH than did bone char.

Decolorizing resin treatment decreased the sensitivity. You would expect that liquor that has been ion exchanged might be harder to decolorize than the original. This is because you have taken out what appears to be the colorant most easily absorbed by char, the pH sensitive color. It didn't work out that way. The treated liquor decolorized quite well with a lower percent color remaining than did untreated liquor. Of course, in ion exchange processes you are doing more than just taking out colorants; you are changing the ash constituents, which are very important too.

T. M. Rinehart (Atlas): Dr. Smith, you showed the increase in the sensitivity of color as your displacements increase. Do you think this would be due to adsorption or displacement of the adsorbed material?

N. H. Smith: The net rate of color removal is essentially the difference between the rates of adsorption and displacement which occur continuously and simultaneously. The increase in pH sensitivity is due to the differences between types of colorants in their net rates of adsorption. Both of the possibilities you mentioned may be responsible for this difference.

T. M. Rinehart: Do you think you are manufacturing any pH sensitive material?

N. H. Smith: I don't think so. If we run the column long enough, the sensitivity drops back down again. Also the amount of sensitivity shown by the effluent depends on how much we start with.

If we have relatively pH insensitive colors to start with, we don't get as high a sensitivity in the effluent.

F. G. Carpenter(SURDD): We found a few years ago that we could separate color into two fractions, an ionic and a nonionized color.

In your statement that when you deionized the sugar you seem to take out the pH sensitive part, that would indicate that perhaps the pH sensitive part was the ionic part of the color.

Do you think that we can make this simple correlation?

N. H. Smith: I don't believe it is that simple. Both pH sensitive and pH insensitive fractions may contain ionic and nonionic compounds. The resin I referred to was an anion resin. Cation resin treatment produces the opposite effect: a liquor of increased pH sensitivity which is harder to decolorize by char on a percent color remaining basis.

F. G. Carpenter: I have measured the pH sensitivity of the melanoidin type reaction products, and I agree that they are nil. However, I, for one, found that the caramel colors, that is the color produced by heating under acid conditions, is often extremely pH sensitive.

I might have used slightly different conditions than you did. So it indicates that the colorant is very subtle, and the reaction may proceed in any direction.

N. H. Smith: I think it's rather significant that pH sensitive colors would be made under acid conditions. It may be that this colorant produced under acid conditions and pH sensitive, is destroyed by heat under alkaline conditions. This could account for some of the low pH sensitivity of some raw sugars.

Also, I have used other adsorbents. Powdered nylon is supposed to be specific for polyphenolics. I found that it takes out preferentially the pH insensitive colors.

SECOND SESSION: K. R. Hanson, American Sugar Co., Chairman

CRYSTAL CHEMISTRY OF CALCIUM PHOSPHATES [1/]

by
Walter E. Brown
American Dental Association Research Associate
National Bureau of Standards
Washington D. C.

Calcium phosphates are used extensively in clarification and decolorization of sugars. The chemistry of these processes is complex and relates closely to the physical and chemical properties of the poorly crystallized calcium phosphates that are present. The number of calcium phosphates one is likely to encounter in these processes is surprisingly large. This, plus the fact that the crystallites are very small, makes it difficult to determine which salts are involved and how their properties influence the efficiency of removal of impurities as well as filterability.

It is all too easy to lump the sparingly soluble calcium phosphates into a group and overlook their individual chemical properties; yet in many chemical applications these differences can be of great importance. Another common tendency is to avoid giving the individual calcium phosphate (particularly hydroxyapatite) fixed chemical properties. According to this view, the composition of the salt is thought to vary over wide ranges and concomitantly its chemical properties (e. g., solubility, reactivity) are not assessable. My view is that the variability in composition

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[1/] Cooperative research at the National Bureau of Standards with the American Dental Association; the Army Dental Corps; the Dental Sciences Division of the School of Aerospace Medicine, USAF; the National Institute of Dental Research; and the Veterans Administration.

should be attributed primarily to the presence of mixtures of salts and that the best hope for understanding the properties of the mixtures is to know those of the individual salts. This permits one to take into account such factors as the relative stabilities of the salts under the given circumstances and to predict which crystals will grow at the expense of the others. In extremely finely divided precipitates such as those that occur in the defecation process, surface phenomena undoubtedly influence strongly the apparent composition of the precipitate. This is an extensive subject in itself, however, and cannot be discussed adequately in this presentation.

Table 1 is a list of the seven well established calcium phosphates; an eighth (isoclasite) was reported long ago, but has not been comfirmed. In this discussion, emphasis will be placed on two of the eight, hydroxyapatite and octacalcium phosphate. Some of the more important chemical characteristics of the others will be noted first. Stress is placed, also, on their anomalous properties, because very often these are of importance in understanding the chemical behavior of these salts or in using them in experimental studies.

Dicalcium phosphate dihydrate, $CaHPO_4 \cdot 2H_2O$--Of all the salts listed in table 1, this is the easiest to precipitate in good crystalline form and with the correct composition; it is, therefore, a good starting material, along with recrystallized phosphoric acid and purified calcium carbonate, for the preparation of the other calcium phosphates. It should be stored in the refrigerator, however, because it has been known to revert at room temperature to the anhydrous salt. It usually can persist in dilute aqueous solutions for long periods of time, but at higher temperatures, or in concentrated solutions its reversion is quite rapid. It is metastable with respect to one or more of the other salts under all known conditions, but is capable of maintaining the condition of metastable equilibrium so well that its solubility product (17) is better known than that of any of the other salts listed in table 1. Its most important property relative to the other calcium phosphates could well be its rapid rate of dissolution or precipitation. As a result, it is easily formed even when it is not thermodynamically the most stable salt. Conversely, it is convenient to use for preparing saturated solutions. This is the best way for preparing solutions of precise compositions, and its use for this purpose in crystal nucleation and growth studies is certain to increase. Dicalcium phosphate dihydrate is a layer-type ("sandwich") structure in which Ca-PO_4 sheets are separarated by water molecules (1, 13). Several other salts (8) have similar Ca-PO_4 sheets, but with different fillings between the sheets e. g., $Ca(H_2PO_4)_2 \cdot H_2O$, $CaClH_2PO_4 \cdot H_2O$, $CaBrH_2PO_4 \cdot H_2O$, $Ca_2KH_7(PO_4)_4 \cdot 2H_2O$, and $Ca_2NH_4H_7(PO_4)_4 \cdot 2H_2O$. Almost certainly, all the examples of this type of structure are not known. The other known members of this type are all quite soluble and for this reason complicating interactions (such as layer-type intracrystalline mixtures) between them and dicalcium phosphate dihydrate are unlikely. If there prove to be other sparingly soluble compounds of this type, then such complications are to be expected.

Table 1. --Sparingly soluble calcium phosphates of interest to sugar purification [1]

Basic Calcium Phosphates	Reference
Dicalcium phosphate dihydrate, $CaHPO_4 \cdot 2H_2O$	1, 13
Anhydrous dicalcium phosphate, $CaHPO_4$	12, 14
Alpha-tricalcium phosphate, α-$Ca_3(PO_4)_2$	15
Beta-tricalcium phosphate, β -$Ca_3(PO_4)_2$	15, 18
Isoclasite, $Ca_2OHPO_4 \cdot 2H_2O$	20
Tetracalcium phosphate, $Ca_4O(PO_4)_2$	5
Hydroxyapatite, $Ca_{10}(OH)_2(PO_4)_6$	14
Octacalcium phosphate, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$	3, 7, 9

[1] The names given are those most commonly used. Several of these are carryovers from the old oxide formulas used in the nineteenth century.

Gypsum has a structure closely anolagous to that of dicalcium phosphate dihydrate, and the mineral ardealite is an example of an intracrystalline mixture of these two salts.

Dicalcium phosphate dihydrate was first thought to be centrosymmetric and its structure was determined on this basis (1). Later, a new analysis of the structure revealed (13) that this is only approximately true. Hydrogen bonding is a major factor in destroying the higher symmetry. The role of the protons in this crystal is not fully understood.

Anhydrous dicalcium phosphate, $CaHPO_4$. --In contrast to the dihydrate, this salt appears to be difficult to prepare in good crystalline form and with stoichiometric composition. At room temperature the kinetics of its formation apparently are so unfavorable that the dihydrate is usually the one to precipitate. On the other hand, its thermodynamic stability is such that it is likely to be present in systems at higher temperatures, in concentrated solutions, or after longer periods of reaction. The first two of these conditions--high temperatures and concentrations--are met in the refining of sugar solutions, and, therefore, this salt may have some importance in the defecation process. The other phase likely to be stable under these conditions is hydroxyapatite. Which of the two is the more stable depends on the composition of the solution. In dilute solutions, anhydrous dicalcium phosphate is the more stable if the pH is less than about 5 and hydroxyapatite is more stable at higher pH values (2). The dividing line between the field of stability of dicalcium phosphate and hydroxyapatite depends on the concentrations of anions and cations other than calcium and phosphate and on the concentration (i. e., chemical potential) of the water. If one writes the reaction for the hydrolysis of dicalcium phosphate to hydroxyapatite $10CaHPO_4 + 2H_2O = Ca_{10}(OH)_2(PO_4)_6 + 4H_3PO_4$, it is seen that a reduction in the activity of water by high concentrations or sugar would drive the reaction to the left. This would have the effect of increasing the field of stability of anhydrous dicalcium phosphate so that the dividing line would be at a higher pH. It should be of value to know where the dividing line occurs under the conditions used in sugar refining.

Alpha-tricalcium phosphate, α-$Ca_3(PO_4)2$-- It should be noted at the outset that there is no correspondence between this salt and the material given the name "alpha-tricalcium phosphate hydrate" (10), which will be discussed later in connection with nonstoichiometric apatites. Alpha-tricalcium phosphate is a high-temperature product and is not likely to be enountered in aqueous systems. Its field of stability is at temperatures above 1, 100° C., which is higher than those used for preparation of bone char, and it is unlikely to be encountered even there. At one time it was thought that this salt was free of striking anomalies so characteristic of other calcium phosphates. A recent single-crystal X-ray study (5) revealed, however, that the length of the unit cell in one direction was three times the reported value for this salt. Whether this is an indication of the formation of a superlattice, a chance catching of the crystals in the process of a phase transition, or the result of its being nonstoichiometric is not known.

Beta-tricalcium phosphate β-$Ca_3(PO_4)_2$. --The earliest single-crystal X-ray studies of this salt revealed that the unit-cell contents and the crystal symmetry were in disagreement (15). Probably related to this anomaly is the tendency for small cations (e. g., magnesium) to stabilize this salt. This manifests itself by making it possible for this salt to form by precipitation from aqueous solution only when these cations are present, and by raising the transition temperature from the beta to the alpha form when these cations are present in the crystal. This salt may be present in bone char.

Isoclasite, $Ca_2OHPO_4 \cdot 2H_2O$. --This salt was reported as a mineral, but lack of confirmation makes its existence doubtful. It is probable that the mineral was a mixture of a calcium phosphate and a nonphosphatic calcium salt. Omission of the other anion from the analysis gave an apparent high Ca/P ratio. It is mentioned here for two reasons. First, a calcium arsenate of this composition has been reported, and there is a close parallelism between the calcium salts of phosphate and aresenate. Secondly, a surface complex with this Ca/P ratio has been suggested by Rootare, Deitz, and Carpenter (21) to account for some of the properties of finely divided precipitates that give apatite-like X-ray patterns.

Table 2.--Comparison of unit cell dimensions of hydroxyapatite, tetracalcium phosphate, and octacalcium phosphate

Dimension	$Ca_{10}(OH)_2$-$(PO_4)_6$	Ca_8H_2-$(PO_4)_6 \cdot 5H_2O$	Ca_4O-$(PO_4)_2$
a	9.42A.	19.87A.	11.99A.
b	9.42	9.63	9.48
c	6.88	6.88	6.97
α	90°	89° 17'	90° 48'
β	90°	92° 13'	90°
γ	120°	108° 57'	90°

Tetracalcium phosphate, $Ca_4O(PO_4)_2$.--In table 2 are listed the unit-cell dimensions of this salt along with those of hydroxyapatite and octacalcium phosphate (3, 5). It is immediately apparent that the *b* and *c* axes and the enclosed angle α are nearly the same for all three salts. In the case of octacalcium phosphate, this was taken to indicate that it contained a plane parallel to *b* and *c* that was structurally nearly identical to a corresponding plane in hydroxyapatite (7). This was later established through determination of the structure of octacalcium phosphate (3). The structure of tetracalcium phosphate has not been determined, but I believe that a similar situation may exist between it and hydroxyapatite.

The presence of a common structural layer makes possible intracrystalline mixtures because a transition from one lattice type to the other can take place through the common layer with little strain. This will be shown in greater detail for the combination octacalcium phosphate-hydroxyapatite. The most obvious result of intracrystalline mixtures between these three salts is that the compositions can vary continuously from a Ca/P ratio of 8/6 (octacalcium phosphate) through 10/6 (hydroxyapatite) to 12/6 (tetracalcium phosphate) I believe this is a major cause of compositional variations in apatitic materials, and that this accounts for mixtures such as those for which the term "alpha tricalcium phosphate hydrate" was invented. It accounts also for the formation of pyrophosphates when these materials are heated to circa 600° C., as first reported by Gee and Deitz (11). Octacalcium phosphate forms pyrophosphate when heated. Its existence as a separate phase (in interlayered mixtures) allows the formation of pyrophosphate as long as the temperature is low enough to prevent reaction of the pyrophosphate with the adjacent hydroxyapatite layer to form tricalcium phosphate. Unless one assumes heterogeneity in the sample, it is difficult to account for the formation of pyrophosphate in any preparation with a Ca/P ratio greater than that of tricalcium phosphate.

Tetracalcium phosphate has not been found in precipitates from aqueous solutions; it has been found only in high-temperature preparations. We should not close our eyes to the possibility, however, that it may be a component in apatitic precipitates. In the absence of moisture it could form in the revivification of bone char.

Hydroxyapatite, $Ca_{10}(OH)_2(PO_4)_6$.--This compound is the prototype for the principal crystalline material in bone char and probably also for the final precipitate formed in the defecation step. It is important, therefore, to know its behavior at high temperature and in aqueous solutions. Here its structural relationships with tetracalcium phosphate and octacalcium phosphate become important.

Figure 1 shows two unit cells of hydroxyapatite projected down the *c* axis. The hexagonal arrangement of 6 of the calcium ions and the 6 PO_4 ions expresses the symmetry of the crystal. The 2 pairs of superimposed calcium ions in the acute corners of the unit cell, along with the 6 in the hexagon, total 10 calciums in the unit cell. If the pairs of calcium ions in adjacent cells were drawn in, then these calciums would also reveal a hexagonal arrangement. In the center of the hexagons is a pair of hydroxyl ions. To compare the structure of hydroxyapatite with that of octacalcium phosphate, we need to keep in mind only the hexagons that define the sets of atoms, with particular attention to the region with the shaded atoms.

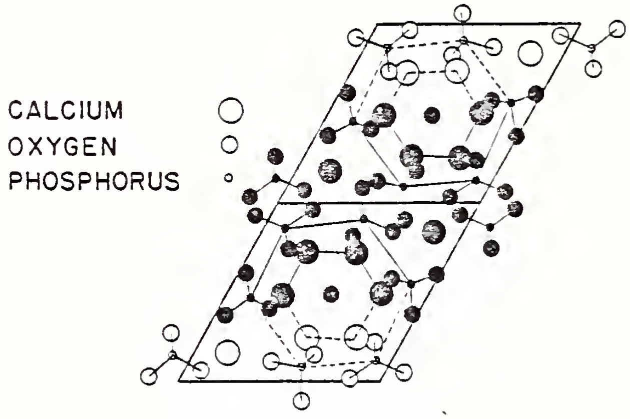

Figure 1.--Structure of hydroxyapatite projected on the c face.

Octacalcium phosphate, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$. --In Figure 2 is shown a projection of one unit cell of octacalcium phosphate down its c axis. It is obvious that portions of the calcium and PO_4 hexagons, slightly distorted, are present here; only four of the six corners of each hexagon are occupied. Also, a pair of calciums, nearly superimposed, are in the acute corner in very nearly the same position as in hydroxyapatite. A water molecule and a phosphate oxygen occupy sites in the center of the partially filled hexagons; these correspond closely to the sites occupied by hydroxyl ions in hydroxyapatite. Although the portions of the two structures represented by the shaded atoms are quite similar; there is no similarity in the positions of the unshaded atoms. In octacalcium phosphate, the region of unshaded atoms contains several water molecules which are, of course, absent in hydroxyapatite.

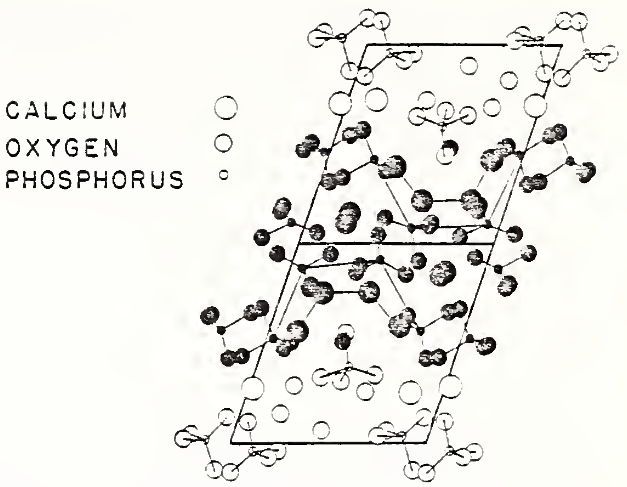

Figure 2.--Structure of octacalcium phosphate projected on the c face.

It has already been mentioned that these two crystalline materials can join at a common plane without much distortion. This is illustrated in Figure 3b which shows a layer of hydroxyapatite two unit cells thick (only the calcium atoms are shown) and a layer of octacalcium phosphate one and one-half unit cell thick. It can be seen that the transition from one crystalline type to the other is continuous. This figure represents a crystallite that would be intermediate in composition between octacalcium phosphate and hydroxyapatite. An X-ray pattern of a "single crystal" that was thought to have a composition intermediate between hydroxyapatite and octacalcium phosphate revealed spots from both types of crystals (9). This is direct proof that both salts were present in the same crystal. Over a dozen other explanations for the nonstoichiometric behavior of apatites have been put forth, but none of these is supported by evidence that is as specific and as direct as that provided by the "single-crystal" study mentioned above.

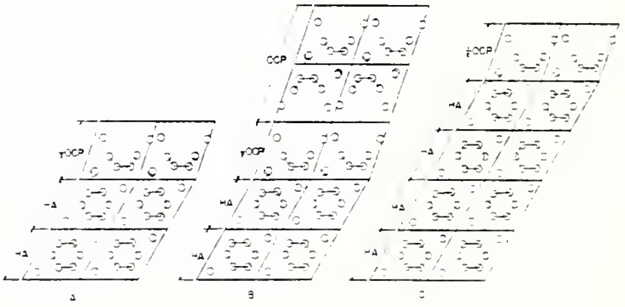

Figure 3.--Mechanism for growth of hydroxyapatite through octacalcium phosphate. Only calcium atoms are shown.

Another effect of the structural similarity between these salts can be hypothesized from physical considerations. In Figure 3a is shown a "crystal" of hydroxyapatite two unit cells thick with a one-half unit-cell thickness of octacalcium phosphate on the surface.

The octacalcium phosphate layer is postulated on the basis that it should reduce the surface energy and, therefore, it would form spontaneously because it would stabilize the crystal. A layer of octacalcium phosphate on the (100) faces of HA crystals would reduce the surface energy for the following reasons:

1. At a certain plane in the outer unit cell of a hydroxyapatite crystal, the structure is the same as that of an octacalcium phosphate crystal (3). Hence, the transition from this layer to the aqueous phase should be the same for the two crystals.

2. Octacalcium phosphate probably has low inter-facial energy because (a) it is a soft crystal and (b) it provides a stable crystallographic transition from an ionically bonded "apatitic layer" to a hydrogen bonded "hydrated layer." The latter should be more compatible with the aqueous phase (because of the similarity in bonding) than the ionically bonded region and would yield a lower interfacial energy.

A stabilizing factor such as this, I believe, is important in bone because it makes possible the extremely small size of bone crystallites, and this in turn gives bone high ion exchange capacity and the ability to remodel rapidly. For similar reasons, a mechanism that stabilizes small crystallites may be important in sugar refining; it would permit the crystallites to have the high specific surface necessary for the removal of impurities.

An important property of octacalcium phosphate is that, like dicalcium phosphate dihydrate, it has a relatively rapid rate of dissolution and precipitation and is frequently found as the product in spite of its metastability.

Mechanisms for formation of hydroxyapatite.--If Figure 3c is compared with 3b one can see that the two diagrams are the same except for changes that have taken place in the octacalcium phosphate layer; water molecules and hydrogen ions have left the site, calcium ions have entered, and the phosphates have been rearranged. This process represents hydrolysis of octacalcium phosphate to hydroxyapatite. It is possible to reproduce this process in the laboratory by simply boiling octacalcium phosphate in water. The resulting hydroxyapatite crystals have the same appearance as the original octacalcium phosphate (i. e., they are blade-like pseudomorphs rather than the common hexagonal needles that are usually associated with hydroxyapatite). This difference in morphology is an important clue for deducing the previous history of apatite-like crystals.

On this basis, hydroxyapatite may be divided into two types:

1. Directly precipitated as hydroxyapatite (hexagonal needles or plates).
2. Hydrolyzed octacalcium phosphate (laths or blades).

To these may be added a third type (4) that would be somewhat intermediate to these in the sense that the initial precipitation in each step is a single unit-cell thickness of octacalcium phosphate and this is followed by its hydrolysis to hydroxyapatite. Since these steps would occur on the unit-cell level, it would be difficult to distinguish between this mechanism and the first when the crystals are very small.

According to the third mechanism, growth of apatitic crystals may take place through a precipitating step alternating with a hydrolysis step. The relative rates of these steps become important. If the precipitation step is more rapid than the hydrolysis step, then octacalcium phosphate, and not hydroxyapatite, is the product. If the two rates are about equal, there will be a tendency for octacalcium phosphate layers to be trapped in the crystal. If the hydrolysis step is too rapid, it may prevent crystal growth by interfering with the precipitation step.

Fluoride is known to induce hydrolysis of octacalcium phosphate to an apatite. This may explain why small amounts of fluoride prevent caries; it assures that the hydrolysis step occurs each time so that none of the less stable octacalcium phosphate is left in the crystal. It also explains why larger amounts of fluoride are harmful--because they may accelerate the hydrolysis step too much, thus preventing good crystal growth. For that reason fluoride in sugar solutions may be an important variable in refining.

With larger crystals, it is known that magnesium ions tend to impede hydrolysis (9). Factors such as this may be important in sugar refining also. A layer of octacalcium phosphate trapped in the hydroxyapatite crystal may subsequently hydrolyze. It can be seen that this process requires a contraction of the unit cell. If closure is not complete, a fissure may be left in the crystal and this could be an important site for removal of impurities from sugar solutions.

The initial precipitate to form in mineralization of enamel is like a ribbon (19). The crystalline prototype for this ribbon appears to be octacalcium phosphate (6). Similar platy crystallites appear to be formed in bone. The formation of platy octacalcium phosphate crystallites as the initial precipitate may be common to many other processes. If this were true for the defecation step, it would be of significance because it would provide a material with an extremely high surface area for adsorption. If octacalcium phosphate is the initial precipitate followed by hydrolysis to hydroxyapatite, it is unlikely that the hydrolysis will yield a precipitate with a significantly lower surface area because the hydrolysis occurs in situ. If some other salt, e. g., a dicalcium phosphate, forms first and then dissolves to reprecipitate as hydroxyapatite, there may be a considerable reduction in specific surface. The low rate of dissolution of the initial precipitate will lead to a relatively slow growth of the hydroxyapatite crystals. Generally, crystals that grow slowly tend to be larger than those precipitated rapidly.

Summary

Clearly, the defecation step, even though it appears to be a simple process, may really be quite complex. Two or more products may form simultaneously. These may be followed by a sequence of other reactions. One must take into account the properties of at least six calcium phosphates that could form in this precipitation. Such properties include rates of dissolution and precipitation, thermodynamic stability (and metastability), ability to form intracrystalline mixtures, and the influence of other components (e. g., F^- and Mg^{++}) on their stability.

Surely, the nature of the precipitate is important in controlling the efficiency of the process. Up to this time, the process has been developed largely along empirical lines. A physico-chemical study appears to be the most promising means for gaining an understanding that would be the basis for further improvements in the process.

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DISCUSSION

M.S. Bhangoo (SURDD): Dr. Brown, we have been trying to prepare some pure hydroxyapatite in the laboratory. We find that it is most difficult to both prepare it and to be sure it is pure. Do you have any suggestions how we can prepare some hydroxyapatite?

W.E. Brown (National Bureau of Standards): Well, this is just another illustration of the difficulties involved. It's hard, very hard, to make pure hydroxyapatite. We have been struggling with this for a very long time, and as an example, we are now able to prepare crystals that when you look at them under the microscope, they look nice, platy, and homogeneous. If you put them in the right index of refraction oil, you discover that you can see a center portion, which you should not be able to see. This means that the material inside is of a lower refractive index and that you really have two crystals.

N.H. Smith: Would someone care to comment on the contribution the hydrolysis of dicalcium phosphate to hydroxyapatite makes to the production of low pH's in the effluents from bone char.

F.G. Carpenter (SURDD): Although that might be a tempting reaction because of the appearance of acid as one of the products, I suspect that it is far more complex than that. The kilning probably changes all the dicalcium phosphate to hydroxyapatite. The hydroxyapatite is the reason bone char does a pretty good job of buffering.

Now, when it does a poor job, it is probably a combination of many reactions, one of which certainly involves carbonate. I am not prepared to say how much this reaction contributes, but I would guess not very much.

Y. Yankelevits (SuCrest): Dr. Brown, you mentioned that in order to form pure hydroxyapatite from dicalcium phosphate you add fluoride as a catalyst or fluoride would speed up this reaction.

Could you tell me what form of fluoride you add and whether you'd form hydrofluoric acid in the process?

W.E. Brown: The fluoride that was added in Dr. Newesely's experiment was a very small amount, sodium fluoride, I assume. His concentrations were of the order of 0.03 molar for the calcium and phosphate ion, whereas he added enough fluoride so that he had only one fluoride for every thousand or five thousand phosphate ions. So you see that was extremely dilute. It's lower than in water.

As for the reaction, fluoride can react with hydroxyapatite in two ways: one, to form calcium fluoride, and if that happens, you get phosphoric acid; the other reaction is that you get fluoride apatite, and this produces very little change in the over-all pH.

I might add that the purpose of this equation is to show that in sugar solutions, the dicalcium phosphate may be more stable at higher pH's than we might expect from experiences with other solutions.

L. F. Martin (SURDD): President Gillette and our collaborators in the cane sugar refining project, I must take just a minute or two to say how glad I am in telling you why I am particularly happy personally, that we have assembled today and that this project is going forward. We were recalling at lunch that it was Dr. Binkley who, about 4 years ago, was the first one to tell me that the Bone Char Project at the Bureau of Standards was to be discontinued because of a change in the policy of the Bureau. And it happened I was on my way to Washington for an appointment with Vic Deitz the next day, and we had been collaborating with Vic and his group and with Frank Carpenter since the New Orleans Technical Session on Bone Char in 1953. And I pointed out to Vic that the Department of Agriculture policy welcomed industrially sponsored projects. We think this is an effective way to work, and we decided that something should be done to have the Department invite the refiners to continue the project, to continue these excellent sessions, and to move in with us. So we just planted this seed, and then it was watered and the plant was nurtured by Dwight Gillette, Joe Bemis, and others who joined in. And therefore, I am happy, and it's a source of personal gratification for me that I am looking at you today and to be able to present a report at the session.

A METHOD FOR ANALYSIS OF THE REDUCING SUGARS OF MOLASSES

by
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(Presented by L. F. Martin)

Analysis of reducing sugars by copper reduction methods such as Lane and Eynon has the disadvantage of grouping all the sugars and other reducing substances together as "invert." Effective methods have been sought for the quantitative analysis of the four isomers which constitute the reducing sugar fraction of molasses or raw sugars. The fraction was isolated by chromatography on carbon columns eluted by controlled gradients of alcohol concentration. This work has been facilitated by use of the highly sensitive ETL-NPL automatic polarimeter for monitoring development of the chromatographic separations of reducing sugars, and for precise determinations of polarization in conjunction with chemical estimations of the reducing sugars in the fractions obtained.

Introduction

The reducing sugars in raw and clarified sugarcane juices are glucose and fructose in approximately equal proportions constituting "invert," but Lobry de Bruyn observed that the transformation which he had discovered in 1895 (1) occurred during concentration and crystallization of sugar, resulting in the presence of mannose and a fourth isomer, now identified as psicose, in final molasses. Contemporary analytical research by Pellet (2) showed that the concentration of mannose ranged from traces to as much as 1 percent of the solids in molasses from Louisiana, Egypt, and other sources at that time. General adoption of the method of Lane and Eynon (3) for estimation of "reducing substances" as invert has obscured these early investigations, and the validity of Lobry de Bruyn's observation has been questioned for lack of supporting analytical data. Unfortunately, the brilliant and painstaking analytical work of Pellet was recorded in a journal, now discontinued, that is not readily accessible to sugar chemists.

Attention has been redirected by more recent investigations to the disparity of the proportions of reducing sugars in both factory and refinery molasses (4) from the equimolecular ratio found in juices. Kelly (5), using conventional methods, found that fructose predominated in four of five samples of Australian molasses. Foster and Marsh (6) isolated the sugars chromatographically and determined that fructose, with which mannose was included by the procedure which they employed, is present in higher concentrations than glucose in all of the molasses which they analyzed.

This inquiry has been pursued in our Laboratory by chromatographic methods for isolating the true reducing sugars, and it has been shown that fructose plus mannose is present in greater proportions than glucose in molasses from various sources, with the exception of that obtained from juices purified experimentally by ion-exchange prior to concentration (7). In collaboration with Binkley (8), the presence of mannose noted by Lobry de Bruyn has been confirmed and this product of transformation of the naturally occurring sugars has been shown to constitute as much as 1 percent of solids in molasses produced under modern processing conditions. The formation of psicose also has been established in these investigations.

Effective methods have been sought for the quantitative analysis of the reducing sugar fraction of molasses isolated by chromatography on carbon columns eluted by controlled gradients of alcohol concentration (9). This work has been facilitated by use of the highly sensitive ETL-NPL automatic polarimeter for monitoring development of the chromatographic separations of reducing sugars, and for precise determinations of polarization in conjunction with chemical estimations of the reducing sugars in the fractions obtained. Determination of glucose specifically is possible by the enzymatic method employing glucoseoxidase (10). An accurate measure of the total reducing sugars is obtained by the Somogyi cuprimetric procedure as modified by Heidt, et al. (11). Combined with precise polarizations afforded by the ETL-NPL instrument, these chemical determinations provide independent analytical measurements from which estimates of the percentages of reducing sugars in the mixture obtained from molasses may be calculated.

The complexity of the reducing sugar fraction which constitutes about one-fifth of the final molasses solids affects the crystallization of sugar, and the quality of raw sugar obtained. The extent of the transformation of naturally occurring glucose and fructose to yield mannose and psicose is a measure of degradation products affecting the quality of raw sugars. Suitability of molasses for particular fermentation processes is dependent upon the nature of the reducing sugars present, and estimates of the composition of this fraction of the product will be useful in expanding its utilization.

Principle of the Method

Quantitative separation of the reducing sugars from sucrose, nonsugar reducing substances, and other optically active or interfering constituents of molasses is effected by adsorption from water on a carbon-Celite column, and selective desorption by increasing concentrations of ethanol in water. The alcohol concentration gradient applied for elution produces only partial fractionation of the reducing sugars, but makes it possible to collect the total fraction in a volume of less than five displacements, which in the columns used (to be described later) is less than 100 ml. In this volume the concentrations of individual sugars, obtained from approximately 1 g. samples of molasses are sufficiently large to provide accurate measurements of polarizations by the ETL-NPL instrument. The small 0.2 dm. sample cell of the polarimeter requires only 10 to 15 ml. of the solution for complete displacement of its contents to record accurately rotations of 0.005° to 0.015°, with a sensitivity of 0.0002°.

Glucose in the mixtures is determined independently by the polarimetric technique developed for measuring the change in rotation from its oxidation to gluconic acid by the specific enzyme, glucose oxidase (12). The analytical procedure consists in the determination of total reducing sugars and of optical rotation before and after oxidation of the glucose by glucoseoxidase. Knowing the specific rotations of all the products involved and using a copper reduction method where all the sugars have approximately the same reducing effect on copper, the amounts of glucose, fructose, and mannose can be determined. The specific rotation (13) of glucose $[\alpha_G]$ at 20° C. and at a mercury wavelength of 546 millimicrons (this condition used in all measurements of this report) is 62.3°. That for gluconic acid $[\alpha_A]$ was measured in this laboratory as 15.3°.

The rotation α, of any solution is related to the specific rotation $[\alpha]$, the tube length L, dm., and the concentration given as N g. of substance per V, ml. by the relation

$$\alpha = \frac{[\alpha]NL}{V} \qquad (1)$$

The difference in rotation of a solution before, α_W, and after, α_0, oxidation is therefore given by

$$\alpha_W - \alpha_0 = \frac{L}{V}([\alpha_G]\ G - [\alpha_A]\ A) \qquad (2)$$

where G = grams of glucose and A = grams of gluconic acid.

Since 1 mole of glucose yields 1 mole gluconic acid:

180.16 g. glucose ⟶ 196.16 g. gluconic acid (3)

$$\alpha_W - \alpha_0 = \frac{L}{V}\left([\alpha_G]\ G - [\alpha_A]\left(\frac{196.16}{180.16}\ G\right)\right) \qquad (4)$$

Since all solutions were made up to V = 100 ml. and the same cell length of L = 0.2 dm. was always used, the amount of glucose can be expressed as

$$G = \frac{\alpha_W - \alpha_0}{0.092} \qquad (5)$$

The change in rotation of 1 g. of glucose in 100 ml. of solution measured in a 0.2 dm. polariscope tube was experimentally found to be 0.092°, which is in agreement with the calculated value for the stoichiometric oxidation of glucose.

Amounts of fructose and mannose then may be calculated from the remainder of the net polarization and the remainder of the total reducing sugars determined by the microcuprimetric method of Heidt (11), after deducting the values of these quantities attributable to glucose. A convenient equation for calculating the amount of fructose may be derived as follows:

Let S = total reducing sugars (g.). (Details of this measure are given later.)

F = fructose (g.)
M = mannose (g.)

Assuming that glucose, fructose, and mannose are the only sugars present in significant quantities.

$$S = G + F + M \qquad (6)$$

The specific rotations for fructose and mannose were measured in this laboratory for the conditions and concentration range applicable to this work as follows:

$$[\alpha_F] = -104.6$$
$$[\alpha_M] = +\ 17.7$$

The rotations of solutions of these substances under the same conditions of measurement as above are:

$$\alpha_F = \frac{L}{V}\ [\alpha_F]\ F = (0.002)\ (-104.6)\ F$$

$$= -0.2092\ F \qquad (7)$$
$$\alpha_M = +0.0354\ M \qquad (8)$$
also $\alpha_G = \ 0.1246\ G$ (9)

The rotation of the mixture can be expressed as the sum of the rotations of the components

$$\alpha_W = \alpha_B + \alpha_F + \alpha_M \qquad (10)$$

By substituting for the α's in equation 10 with equations 7, 8, and 9, and then eliminating M from the resulting equation and equation 6, one can arrive at:

$$F = -\ 4.087\alpha_W + 0.3647\ G + 0.1447\ S \qquad (11)$$

It should be noted that in real samples the first term is actually positive because α_W is always negative because of high negative specific rotation of fructose.

Lastly, the mannose is calculated by

$$M = S - (F + G) \qquad (12)$$

Effect of the Presence of Psicose

Lobry de Bruyn, in the course of his studies of the transformation of glucose under alkaline conditions, investigated the formation of a fourth isomer that could be predicted from possible configurations of the sugars. He obtained indirect evidence for the presence of such a ketose, which he termed pseudo-fructose, in the transformation products. However, he says that "we begin by declaring that its existence is not considered by us as being definitely established but merely that it is rendered quite probable" (14). Almost half a century later this sugar, which had by then been prepared synthetically and characterized as psicose, was isolated from the residual constituents of molasses after fermentation by Sattler and Zerban (15). Psicose has been detected in molasses from various sources, as well as in the reducing sugar mixtures separated by carbon column chromatography in this work, by investigations employing appropriate chromatographic methods (6, 8).

None of the research on isolation or synthesis of psicose has resulted in preparation of a crystalline product, but low dextrorotatory values have been recorded for the optical activity of the sirups or amorphous material obtained. From these data its specific rotation may be estimated roughly as +5.5° for the mercury 546 wavelength, and its reducing power may be assumed to be approximately that of the other sugars in the mixture. Assuming that psicose is the only isomer other than glucose and fructose in a mixture of reducing sugars separated from molasses, an equation similar to equation 11 may be derived for calculation of the weight of fructose present. For an actual sample containing about 2 percent on solids of the third sugar, this calculation yields 74.5 mg., or 10.03 percent fructose, contrasted with 76.4 mg., 10.29 percent, based on mannose. Corresponding values for the third component are 15.7 mg., 2.12 percent mannose, and 17.6 mg., 2.37 percent psicose. The maximum error in estimating fructose in a four component mixture thus would be 2.5 percent. Evidence from ionophoresis is that all samples examined by this technique contain psicose as well as mannose, but the latter is present in larger amounts. On the assumption that the ratio of mannose to psicose is 4/1, the value obtained for fructose would be only 0.5 percent too high in the sample under consideration by calculation from equation 11, neglecting the presence of the small proportion of psicose. This example was chosen because the particular molasses contains a relatively large amount of reducing sugars other than glucose and fructose; the error would be less in samples containing smaller percentages of the sugar transformation products.

Isolation of Reducing Sugars

The experimental arrangement of the carbon column for adsorption, elution by an alcohol-water concentration gradient, and monitoring of the eluate fractions through the polarimeter are depicted schematically in Figure 1.

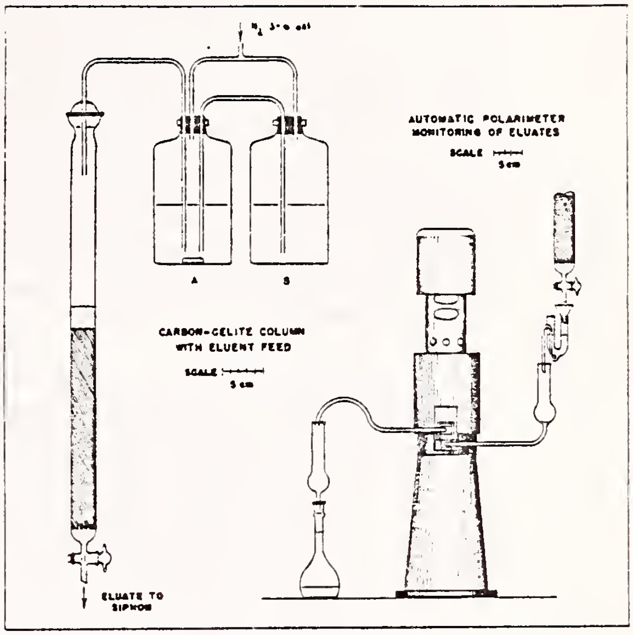
N_2 3-5 psi
A
B
CARBON-CELITE COLUMN WITH ELUENT FEED
SCALE
5 cm
AUTOMATIC POLARIMETER MONITORING OF ELUATES
SCALE
5 cm
ELUATE TO SIPHON

Figure 1.--Apparatus for chromatographic separation of reducing sugar fraction by alcohol concentration gradient elution from carboncelite column, and monitoring of eluate by the automatic polarimeter.

The 2.5 cm. i.d. column is packed with a mixture of equal weight of untreated carbon Darco G-60 and acid washed Celite 535. Of the materials, 20 g. of each are mixed to a slurry in water in a high speed blendor, poured into the column, and packed under pressure. The total 40 g. occupies a column height of 20 to 25 cm. which is suitable for most molasses samples; some longer and shorter columns were used, employing total amounts of the adsorbent mixture ranging from 60 to 30 g., particularly for samples of unusual molasses from which the reducing sugars could not be separated completely from sucrose on columns of standard lengths. Satisfactory results were obtained with 40 g. or 45 g. columns for all of the molasses for which analyses will be given in this report.

No clarification, deionization, or other pretreatment of the molasses is necessary for effective separation of reducing sugars by the carbon column technique. Samples of whole molasses closely approximating 1 g. were weighed accurately to 0.1 mg. and dissolved in 10 to 15 ml. of water for application to the column. A minimum amount of water was used to transfer the material quantitatively, and the sample was forced into the top layer of adsorbent under about 10 p. s. i. of nitrogen gas pressure. Samples of this size provide optimum loading of the standard column. The apparent advantage of using larger samples to obtain higher concentrations of the sugars in the solution collected for analysis is not realized since the volume of eluates containing these sugars exceeds 100 ml., and trailing fractions of the hexoses may be overlapped by the initial elution of sucrose. After rinsing the sides of column with a small volume of water that is forced into the carbon, the eluant supply is connected and flow is maintained under 3 to 6 p. s. i.

The simple arrangement of two 1 l. bottles interconnected by a siphon provides the gradient of increasing alcohol concentration as described by Alm (9). The optimum gradient was obtained with approximately 500 ml. absolute alcohol in bottle B feeding into 500 ml. of water in bottle A in which a magnetic stirrer insures mixing of the solvents. The concentration gradient is linear with bottles of equal diameter. Flow rates varied, being initially of the order of 1. 5 ml. /min., decreasing to less than 1 ml. /min. as elution proceeded with increasing concentration of alcohol.

The automatic polarimeter can follow changes in polarization with appearance of the sugars in a continuous flow of eluate, but the magnitude of the net rotation obtained by integrating the polarization recorded in this manner is too high. A flow pattern is established which results in a higher concentration of the sugar passing through the narrow beam in the center of the instrument sample cell. The small, bifurcated siphon shown in Figure 1 delivered fractions of approximately 5 ml. intermittently to the funnel supplying the cell. This volume is ample for displacement of successive fractions as the retention volume of the 0. 1 dm. cell used in monitoring is only 1. 2 ml.

Figure 2 illustrates the separation of known weights of pure glucose, fructose, and mannose from a mixture with approximately 0. 26 g. of sucrose monitored by the recording polarimeter. The hexoses were recovered quantitatively in the eluent volume of 60 ml. required for desorption, and collection of the reducing sugar fraction required 3. 5 hours. The initial fractions are levorotatory, showing that partial fractionation occurs with fructose being desorbed first, followed by dextrorotatory fractions containing larger proportions of glucose and mannose. The important property of gradient elution is that of containing the total hexoses in as small a volume of eluate as possible rather than fractionation of the individual sugars.

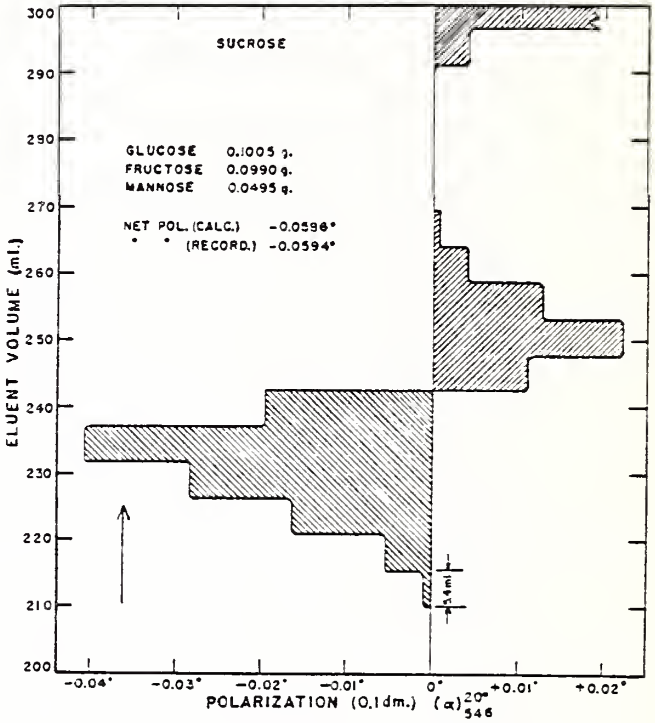

Figure 2.--Plot of chart record for separation of a known mixture of pure reducing sugars from sucrose.

Determination of Glucose

Glucose is determined independently by oxidation with the specific enzyme, glucose-oxidase, and measurement of the resulting change in polarization of the solution with the automatic instrument. The conditions employed eliminate measureable changes in rotation from the slow oxidation of mannose by the enzyme (16). The colorimetric method (10) is not satisfactory for analyzing these mixtures because high results are produced by oxidation of mannose under the conditions required for developing the color. The polarimetric measurements yield more precise results because it is applied to an undiluted aliquot of the sugar solution, whereas colorimetry requires dilution to 0. 05 mg. per ml., or less. A commercially available, highly purified enzyme preparation[1] is used because its high activity insures complete oxidation of glucose in 30-45 minutes with only 1 mg. of enzyme for the quantities of the sugar in the sample aliquots. Duplicate determinations were made with 30 ml. aliquots of the reducing sugar eluate made to exactly 100 ml. at 20° C.

Oxidation is carried out in 100 ml. volumetric flasks fitted with glass stoppers through which a tube extends near the bottom for introduction of oxygen. To each 30 ml. aliquot is added 10 ml. of 0.6 M potassium phosphate buffer adjusted with citric acid to pH 6.35, and 1 ml. of the enzyme solution containing 1 mg. per ml. The flasks are attached to a wrist-action shaker modified by replacement of the solid rocker arms by tubes which serve as manifolds for supplying oxygen gas under slight positive pressure. Air is displaced from the flasks by bubbling oxygen through for about 30 seconds after which they are closed and shaken for 45 minutes. Upon completion of the oxidation the solutions are made up to exactly 100 ml. at 20° C., and filtered through dry filter papers into dry flasks to remove a small amount of precipitated enzyme material which would interfere with polarization in the automatic instrument. The polarization of the unoxidized sugar mixture, α_W, is determined on a portion of the undiluted 100 ml. of eluate from the column.

Additional details of the procedure will be described elsewhere (12). It has been determined that these conditions for oxidation of glucose lead to the stoichiometric production of gluconic acid, and that the measured difference in polarizations of the oxidized and unoxidized solutions is directly proportional to the amount of glucose present. The applicable factor determined experimentally to be 0.046°/g. 100ml. 0.1 dm., agrees with the value calculated from measured specific rotations of the compounds. Rotations of larger magnitude giving more accurate measurements are obtained by polarization in a 0.2 dm. cell for which the factor is 0.092° used in derivation of the equation for calculating the fructose in these analyses.

Determination of Total Reducing Sugars

The most satisfactory method for determining total reducing sugars in the concentrations and volume of eluate obtained from the carbon column is the microanalytical modification of the Somogyi procedure developed by Heidt, et al. (11), although the values obtained for mixtures of the sugars are less accurate than those attainable in determinations of the individual sugars. Small amounts of sample are required and the method is convenient because a number of samples may be analyzed simultaneously. The published method gives cuprous oxide yield factors, expressed as moles cuprous oxide, Cu_2O per mole of sugar, ranging from 4.04 for mannose, 4.29 for fructose, and 4.41 for glucose when these sugars are determined separately. The factors were determined by statistical analysis of their data to be accurate within ± 0.5 to 1.0 percent. The factors are the slopes of linear standard curves which were plotted to correct automatically for a small loss of cuprous oxide before titration for each of the sugars, applicable under the conditions of the procedure adopted for liberation of iodine and titration with thiosulfate.

It was necessary to establish that the yield of cuprous oxide from oxidation of a mixture of the sugars is equal to the sum of the yields produced by the amounts of each of the individual sugars present. The only modification of the titration procedure adopted for this work was immediate cooling of the oxidized solutions in an ice bath, and rapid addition of the two reagents for liberating the iodine by two persons working in unison. This eliminated significant loss of cuprous oxide before titration and yielded linear standard curves for individual sugar passing through the origin as shown in Figure 3. The curve S in Figure 3 was plotted from experimental determinations on aliquots of a mixture of the sugars in the relative proportions indicated. Its slope, 4.25, agrees satisfactorily with the cuprous oxide yield factor 4.19, calculated as a weighted average yield from the amounts of the individual sugars. A large scale plot of this standard curve was used in calculating the total reducing sugars from the thiosulfate titers.

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[1] Produced by Worthington Biochemical Corp., Freehold, N.J., as G.O.P.-X1403.

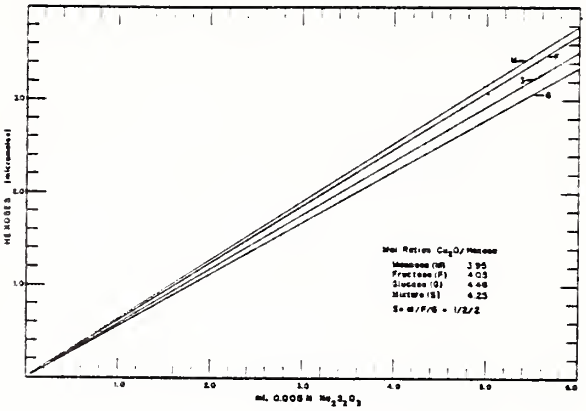

Figure 3.--Standard curve for determination of total reducing sugars by the modified Somagyi method (11).

The best concentration range for this determination is from 0.5 to approximately 2.0 micromoles of sugar per ml., giving thiosulfate titers ranging from 0.8 to 3.5 ml. For samples estimated to contain more than 0.15 g. of reducing sugars, 3 ml. of the original solution was diluted to 50 ml. for the determination; for smaller amounts the dilution was 2 ml. to 25 ml. For each determination three or more aliquots of the diluted solution, ranging from 1 to 5 ml., were oxidized by the standard 5 ml. of the copper reagent. Agreement was always satisfactory for at least two such aliquots in the best range of the method. As optimum heating times in the boiling water bath given in the published procedure range from 10 min. for fructose to 30 min. for mannose, a compromise was adopted by heating the sugar mixtures for 25 min., then cooling and titrating immediately.

Molasses Samples

The molasses chosen for analysis were from quantities that had been collected for other purposes and were catch samples rather than composites. Four were refinery final molasses of which two were from plants employing the carbonation process, while the others were from a refinery operating with phosphate-lime in the Williamson clarification process, and from a large refinery using minimum clarification and primarily kieselguhr filtration. Three samples of blackstrap molasses from Louisiana raw sugar factories also were analyzed. Five of these samples yielded concardant analytical data from duplicate determinations that could be calculated in terms of the expected percentages of glucose, fructose, and mannose with minor proportions of psicose. The results for one refinery molasses and for one sample of blackstrap indicated the presence in the reducing sugar mixture of additional optically active and probably nonreducing substances. It is believed that these two samples are abnormal as the results of contamination or spoilage of the blackstrap and deterioration of the raw sugar processed by the refinery from which final molasses was obtained. Examination by ionophoresis, together with polarimetric and reducing sugar determinations, provide evidence that the abnormal constituent is a fructose anhydride. The results for these samples will be considered separately from those for the five normal molasses.

Results

Separation of the reducing sugar fraction from a normal refinery molasses sample depicted by the plot of the chart record in Figure 4 illustrates the similarity of the pattern to that obtained with the mixture of pure glucose, fructose, and mannose. There is no essential difference between refinery molasses and blackstrap as shown by the pattern obtained from a Louisiana raw factory sample shown in a similar plot in Figure 5. The proportions of the individual reducing sugars in the final molasses of a refinery are determined largely by those in the molasses film on raw sugar separated in the initial stage of processing by affination, therefore it is not surprising that the refinery product is similar in composition to factory blackstrap.

Calculation of the percentages of the individual sugars for one sample is presented in detail with the data obtained from an individual determination to illustrate the application of equation 11.

Louisiana factory molasses:

Sample weight = 1.0155 g., Solids 80.28 percent

Reducing sugar eluate from standard column in 100 ml.

Pol., recorded = -25.5 mm.: 1 mm. = 1.925° x 10^{-4}

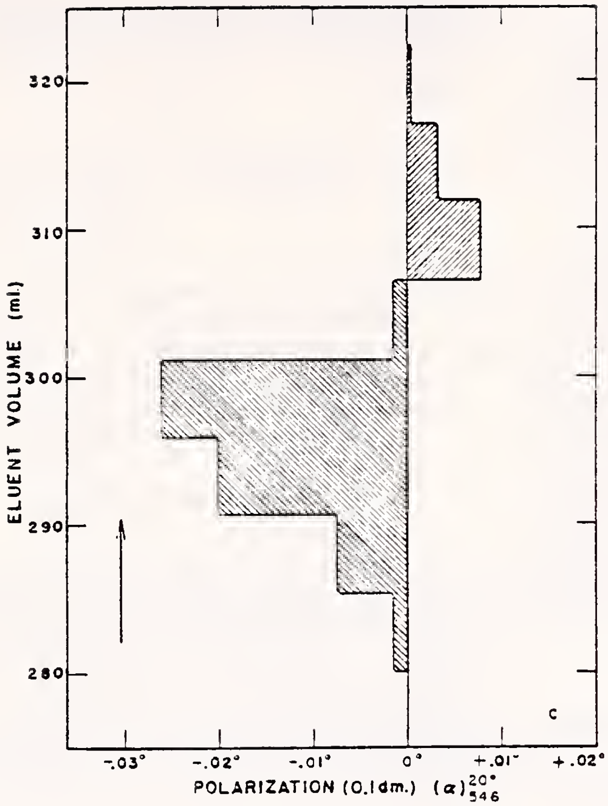

Figure 4.--Plot of chart record for separation of the reducing sugar fraction of a refinery molasses sample

$\alpha_W = -0.00491°$

Oxidation of 30 ml. aliquot in 50 ml.

Pol., recorded = -33.5 mm.

$\alpha_0 = -0.00645° \times 100/30 \div 2 = -0.01075°/$ 100 ml.

$\alpha_W - \alpha_0 = -0.00491° + 0.01075° = +0.00584°$

$G = 0.00584°/0.092 = 0.0635$ g.

Total red.sugar oxidation, 3 ml. aliquot in 50 ml.

Titers, 0.005 N $Na_2S_2O_3$ (ml.):

		△	△ /ml.
Blank	19.75		
Subaliquots 3	17.37	2.38	0.793
(ml.) 4	16.57	3.18	0.795
5	15.68	4.07	0.814

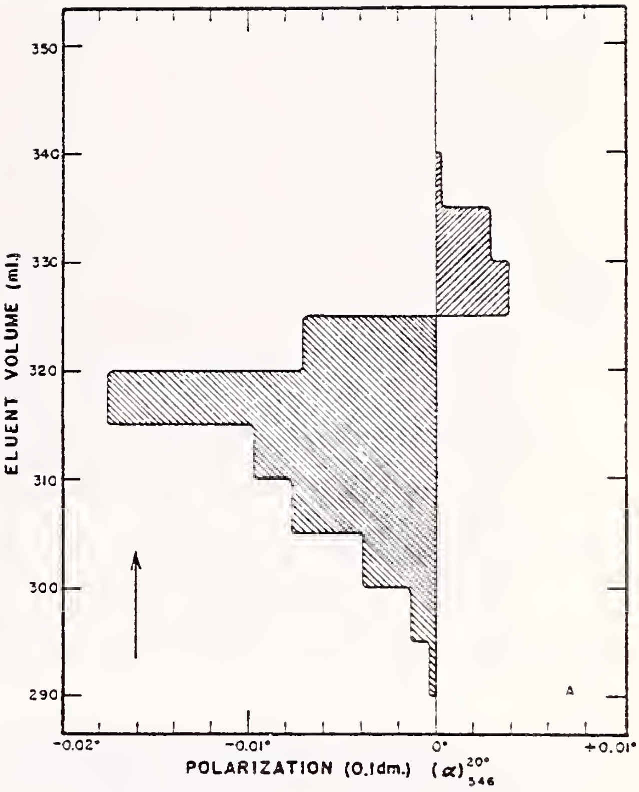

Figure 5.-- Plot of chart record for separation of the reducing sugar fraction of a blackstrap molasses sample.

Sugars calc. from 3 and 4, aver. 0.794 ml.

Sugars, from curve S, Figure 3, 0.47 micromoles/ml.

$S = 0.47 \times 10^{-6} \times 180 \times 50/3 \times 100 = 0.141$ g.

From equation 11:

$F = 4.09 \times .00491 \times 0.3647 \times 0.0635 \times 0.1447 \times 0.141$

F = 0.0642 g. 7.87 percent solids

M = 0.0133 g. (by diff.) 1.63 "

G = 0.0635 g. (direct) 7.79 "

S = 0.1410 g. 17.29 "

Results of the analyses of the five normal samples are assembled in table 1. Duplicate analyses were carried out on four of the samples indicating the reproducibility attainable by this combination of methods, while only a single determination was made on one of the refinery molasses samples. The two samples of blackstrap were found to contain both actual and relative concentrations of mannose several times greater than those found in the three final molasses samples from the refineries. A much larger number and variety of samples would have to be analyzed to establish the significance of this observation. Transformation of the sugars in raw sugar production occurs principally during prolonged heating of concentrated massecuite in sugar boiling. The refinery product is obtained by similar boiling of low grade and affination sirups during which additional mannose may be expected to form and its concentration should be comparable to that in blackstrap molasses. The low concentrations might be attributed to the accumulation of relatively large amounts of glucose and fructose during other phases of the refining process as a result of the inversion of sucrose.

Psicose undoubtedly is present in all of the samples, although it was determined by ionophoresis only in the carbonatation refinery product. Figure 6 is a tracing of the chromatogram showing the presence of this fourth sugar in lower concentration than the mannose, but in definitely detectable amounts with the known electrophoretic mobility of this isomer.

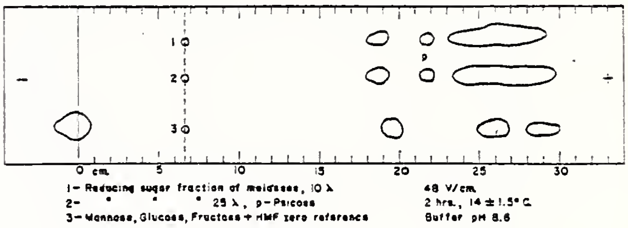

Figure 6.--Tracing of the chromatogram obtained for the reducing sugar fraction of refinery molasses by ionophoresis, showing the presence of psicose as well as mannose.

Abnormal Molasses

Calculation of fructose from equation 11 from the data obtained for one blackstrap and one refinery molasses sample yields negative results. The data and calculations for the refinery sample, which apparently contained more of the abnormal constituent, are presented in detail to show the magnitude of the discrepancy.

Abnormal refinery molasses:

Table 1.--Concentrations of glucose and fructose, and of other reducing sugars estimated as mannose in molasses

Sample[1/]	Solids (pct.)	Sample Weight (g.)	$\overset{\circ}{\alpha}_W$ X10^3	Tot. sugar (S) (g.)	Glucose (G) (g.)	Reducing sugars: Total	Glucose	Fructose	Balance (as mannose)
						(pct. of solids)			
Factory	80.28	1.0155	-4.91	0.1405	0.0635	17.21	7.79	7.79	1.63
		1.0008	-4.51	.1424	.0645	17.73	8.03	7.78	1.92
Fact. (B)	71.53	1.0190	-3.77	.2090	.1048	28.70	14.40	11.51	2.79
		1.1038	-3.58	.2100	.1099	26.61	13.94	10.80	1.87
Ref. (C)	73.97	.9898	-4.91	.1745	.0843	23.83	11.51	10.40	1.92
		1.0045	-4.93	.1760	.0839	23.70	11.30	10.27	2.13
Ref. (P)	74.64	.9978	-6.25	.1653	.0821	22.21	11.03	10.67	.51
Ref. (F)	70.71	1.0398	-5.09	.0964	.0419	13.14	5.70	6.92	.52
		1.0336	-5.04	.0943	.0428	12.90	5.86	6.92	.22
		1.0106	-5.33	.0931	.0419	13.03	5.86	6.95	.22

[1/] (B)=Determination of sucrose by elution from the carbon column and polarization showed this sample to be factory second (B) molasses with a true purity of 66.4.
(C)=Carbonatation; (P)=Phosphatation; (F)=Kieselguhr filtration.

Sample weight = 1.0355 g. Solids, 79.30 percent

$\alpha_W = -0.01280°$

G = 0.0213 g. (direct detn.)

S = 0.0861 g.

from equation 11

$F = 4.09 \times 0.0128 + 0.3647 \times 0.0213 + 0.1447 \times 0.0861$

F (apparent)	= 0.0727 g.
plus G	= 0.0213
	0.0940 g.
Determined S	= 0.0861 g.
Difference	= -0.0079 g.

The abnormal constituent must be levorotatory, increasing α_W, and nonreducing so that it is not determined with total reducing sugars, S. This result is consistent with the presence of a levorotatory compound which does not have reducing power for the copper reagent used to determine total reducing sugar. The net levorotation of the mixture is thus larger than that of the total reducing sugars determined, calculated as fructose. The discrepancy was smaller in the case of the blackstrap molasses which, in one determination, apparently contained only glucose and fructose but no mannose. Additional duplicate analyses of this sample showed that the discrepancy was somewhat greater than the probable experimental error of the determinations, and that the abnormal constituent actually was present.

Examination of these samples by ionophoresis established the presence of glucose, fructose, and mannose. Small amounts of a fourth constituent other than psicose could not be detected without overloading the chromatograms; therefore, the fermentable reducing sugars were removed to a large extent by yeast fermentation and concentration of the remaining constituent. A tracing of the chromatogram obtained for the blackstrap molasses concentrate depicted in Figure 7 provided tentative identification as a levorotatory diheterolevulosan. Evidence for the presence of compounds of this nature in Australian molasses had been found by Foster and Marsh (6) by the chromatographic techniques which they applied to whole molasses. The larger amounts present in the refinery molasses, together with the unusually low values found for both total reducing sugars and for glucose, are indicative of deterioration in transient and storage of the raw sugar resulting not only in further transformation but in rather extensive destruction of the reducing sugars in the accompanying molasses. A sample of this molasses was run completely through the carbon column development to separate the sucrose which was found to be present in a normal concentration.

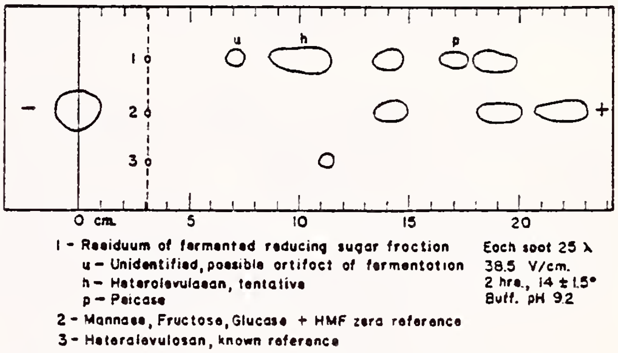
u
h
p
1
2
3
−
+
0 cm.
5
10
15
20
1 - Residuum of fermented reducing sugar fraction
u - Unidentified, possible artifact of fermentation
h - Heterolevulosan, tentative
p - Psicose
2 - Mannose, Fructose, Glucose + HMF zero reference
3 - Heterolevulosan, known reference
Each spot 25 λ
38.5 V/cm.
2 hrs., 14 ± 1.5°
Buff. pH 9.2

Figure 7.--Tracing of ionophoregram of the unfermentable constituents of the reducing sugar fraction of a blackstrap molasses sample, showing the tentative identification of a levorotatory diheterolevulosan.

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DISCUSSION

J. B. Stark (WURDD): Did you run psicose knowns on your columns?

L. F. Martin: You get us the psicose, and we will run it. The problem there is that psicose has never been obtained in crystalline form. Everyone ends up with getting a very little bit by stirring up glucose with ammonia and fermenting the other sugars out, and then they get a syrup. And one article says the specific rotation for the sodium line is 2 degrees plus a minus 2 degrees. Somebody else reports plus six, and one paper says zero. Well, what can you do with this? It would be very interesting to us to get psicose sufficiently pure, know its exact specific rotation in water and particularly what happens in borate buffer. You see, psicose could have practically no rotation in water, but in borate, its rotation might go either way. It might become highly levo. Maybe this Louisiana molasses was loaded with psicose, and when we put it in borate, we got those unusual readings, but we don't know, that will take some further work.

S. Stachenko (C&D): I would like to know what is the alcohol concentration in your 25 ml. fraction where you have your reducing sugars concentrated.

L. F. Martin: Frankly, we don't know exactly, Mr. Stachenko. It's probably not over 10 percent.

S. Stachenko: Does this have any bearing on the enzyme system? Did it have a bearing on the polarization measure having alcohol in the solution?

L. F. Martin: No, we have checked that out. The alcohol does not interfere with the enzyme. Another good point, I am glad you brought it up, we also know that it does not interfere with this copper reduction method of the total sugars, and I don't think we found trouble from alcohol when we ran the colorimetric glucose method, but alcohol in the glucose oxidase method actually helps. When Keilin did his original work, he added alcohol and doubled the stoichiometric amount of oxygen taken up. You see, the hydrogen peroxide oxidizes the alcohol to aldehyde, and it doesn't break up and reform. You get a whole mole of oxygen instead of a half. We think our method of determining glucose is much better because Dr. Teller at Worthington has available a very active highly purified form of glucose oxidase. We could probably oxidize one or two tenths of a gram of glucose with one milligram in this volume we are running.

The alcohol, of course, doesn't have any effect on rotations. It's optically inactive. We have never found even with the highly sensitive polarimeter any effect of alcohol solutions on rotations of sugars.

We do all the work with the 5461 line. That happened to be the filter in the instrument, and we didn't change to the sodium filter for the simple reason the readings are all about the same height at that wavelength, so we get better readings and this helps the whole procedure.

S. Borodkin (American): I was a little bit unclear as to how you separate your fructose from your mannose.

L. F. Martin: You do that by just a little algebra. We first determine the glucose. We also determined by our Somagyi total reducing sugars. We subtract the glucose leaving the amount of fructose plus mannose. We also subtract the polarization of the glucose, giving the polarization of the already known total weight of these two sugars. It is then just like using the polarization on various proportions of invert.

ION EXCLUSION PURIFICATION OF MOLASSES

by

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The separation of ionic from non-ionic substances using ion exclusion is only a little over 10 years old. Ion exclusion purification arose from the observation that at equilibrium the concentration of a strong electrolyte is lower within the aqueous portion of the resin phase than in the surrounding solution. The electrolyte is partially excluded from the water in the resin bead. Non-ionized or weakly ionized compounds generally have a relatively high concentration within the resin bead compared to highly ionized compounds. When the differences in relative concentrations inside and outside the resin bead are sufficiently large, two substances may be separated readily on a resin column.

Essentially ion exclusion purification is a chromatographic technique. The components to be separated are loaded on the column and washed through with water as the eluant. Compounds having a lower relative concentration in the resin bead will be eluted more rapidly and hence separate from components having a higher relative concentration in the resin bead. Non-ionized or weakly ionized compounds may also separate from each other if there is an adequate difference in their relative concentrations between the solution inside and outside the resin beads.

Recovery of sugar from molasses by ion exclusion offers a number of advantages over ion exchange. A cation exchanger is cheaper and more stable than an anion exchanger. Operation near pH 7 precludes sucrose inversion. There is no expense for costly regenerants as water elutes both the sucrose and impurities from the column. Fewer resin beds should be required as only one resin is utilized.

Wheaton and Bauman (1, 2) discussed theory of ion exclusion and its potential applications in 1953. Patents have been issued for the separation of ionic materials having different degrees of ionization (3) and for the separation of certain organic compounds (4). This latter patent (4) describes separations for glucose-acetone and sucrose-glycerol-triethylene glycol. A later article by Asher (5) discusses sugar purification by ion exclusion and gives examples of the separation of salts from dextrose or sucrose. Color and strong ionic materials present in molasses or crude cane sugar were separated from the sucrose. A patent by Wheaton (6) on ion exclusion purification describes similar experiments including treatment of cane molasses to remove ionic compounds. Simpson and Wheaton (7) give a good resume of the effects of a number of variables that influence the efficiency of ion exclusion separation--particle size, flow rate, crosslinkage, and feed volume to column volume ratio--using ethylene glycol as a test compound. Simpson and Bauman (8) describe a procedure for recycling effluents from an ion exclusion separation of ethylene glycol from sodium chloride which could possibly be adapted to sugar liquor purification. A more recent publication by Norman, Rorabaugh, and Keller (9) discusses some of the advantages of ion exclusion as applied to sugar juice purification. They also postulate operating requirements for a continuous Higgin's contactor (10) which might be used to purify sugar beet thick juice diluted to 40 Brix.

None of these papers indicate any separation of reducing sugars, i. e., glucose or fructose from sucrose and one (4) specifically states that glucose cannot be separated from sucrose using ion exclusion. However, Jones and others (11, 12) and Carruthers and others (13) demonstrated the feasibility of separating mono-, di-, and trisaccharides for analytical purposes on Dowex 50 W, (2 percent DVB, 200-400 mesh, Li Form) using columns 100 to 150 cm. long and 1 to 2 cm. in. diameter. These analytical conditions would be impractical industrially. Cations in the sugar liquors would displace lithium and the load and rate of elution are too low.

There is little information in any of these papers to indicate whether the impurities other than salts and color present in sugar liquors, such as amino acids, salts of weak acids, saccharides, and others, may be separated from sucrose by ion exclusion. It seemed possible that, in addition to separating strong salts and color eluted before sucrose, other impurities might be eluted after sucrose enabling further purification. This paper describes a number of experiments using cane and beet molasses that show the separation of some molasses components by ion exclusion.

Materials and Procedures

The resin was Dowex 50 W, 50-100 mesh. The potassium form of the exchanger was used since this form of the resin would most nearly approximate the form that would develop during factory operations on beet molasses. Studies compared the efficiency of X-4 and X-12 crosslinkages at room temperature and at 90° C. Two Pyrex glass columns were joined to coarse sintered glass funnels: One for use at room temperature was 90 mm. tubing, 135 cm. long, containing 6, 500 ml. of resin 115 cm. high; the other was 50 mm. tubing, 115 cm. long, jacketed for operation at 90° C., and containing 1, 800 ml. of resin 100 cm. high. Work with columns of this size is generally considered suitable for scaling up to industrial-sized units. Temperature in the second column was maintained by pumping hot water through the jacket while loading with hot molasses and using hot water for elution. The normal flow rate of 1 ml. /cm.^{2} min. was controlled with a capillary outlet; minor adjustments in rate being made by raising or lowering the effluent reservoir. Load volumes were 15 percent of the resin bed volume, 975 ml. for the large column and 270 ml. for the smaller column. Contraction of 5 to 10 percent in the resin bed during loading and subsequent expansion during elution do not interfere with column operation. Effluent fractions equal to 1/20 of the bed volume were collected and analyzed.

The following general analytical methods were used: Reducing sugars were determined by Munson-Walker copper reduction. Sucrose was determined by invertase inversion followed by copper reduction. Total nitrogen was determined by the Kjeldahl method and amino nitrogen by the Van Slyke procedure. Ash was determined by heating at 550° C. and weighing the residue. Chloride was determined using an Aminco-Cotlove automatic chloride titrator. Color determinations were made with a Beckman Model B spectrophotometer, measuring adsorption at 720 and 425m and reported in arbitrary units.

Experiments and Results

An attempt was made to separate sucrose from glucose at room temperature using the large column with X-12 crosslinked resin. A solution containing approximately 190 g. each of glucose and sucrose in 975 ml. of water was loaded on the column and eluted with water. Two runs were made at flow rates of 1 ml./cm.^{2} min. and one-third that rate. The data for these runs are presented in Table 1 and Figure 1. This and other figures plot V_e/V_T (ratio of effluent volume to resin bed volume) against C_e/C_T (ratio of effluent concentration to original concentration). Results obtained using columns of different sizes may be compared directly on this basis. Although the separation of sucrose from glucose was not complete, nearly 50 percent of the sucrose was in the first three fractions at greater than 80 percent purity. The separation of salt from sucrose described by Asher (5), combined with these results, demonstrates the possibility of separating cane molasses into a salt-rich fraction, a sucrose-rich fraction, and finally an invert-rich fraction.

A series of experiments was conducted using X-12 resin at 25° C. and 90° C. and X-4 resin at 90° C. Dilute beet molasses, 40 RDS, was loaded on the large column containing X-12 resin and washed through with water

Table 1.--Separation of sucrose from glucose at 25° C. using Dowex 50 W(K) X-12

Fraction	Percent of total				Sucrose purity	
	Sucrose		Glucose			
	Run 1	Run 2[1/]	Run 1	Run 2	Run 1	Run 2
8	2.0	2.5	0.2	0.0	92.1	99.0
9	19.7	19.0	.9	.3	95.5	98.6
10	26.3	25.6	6.5	4.0	80.1	86.3
11	23.8	26.5	16.4	13.5	59.1	65.9
12	13.3	15.3	22.9	27.3	36.7	35.6
13	8.1	6.3	21.4	33.5	27.3	15.7
14	4.0	3.5	16.3	17.1	19.7	17.0
15	2.0	1.0	9.5	3.6	17.4	21.1
16	.7	.2	4.0	.5	14.5	32.0
17	.3	-	1.9	-	15.5	-
Total g.	188.4	203.6	188.8	201.6		

[1/] Flow rate of 1/3 ml./cm^2 min.

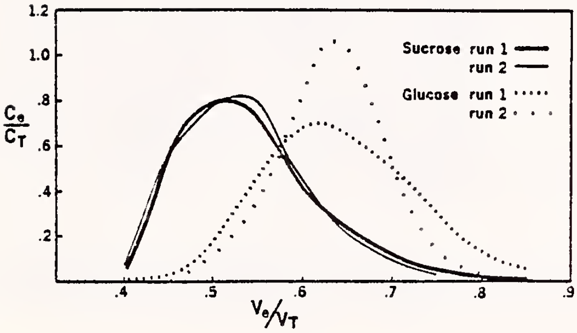

Figure 1.--Separation of sucrose and glucose using Dowex 50W (12 percent DVB, 50-100 mesh, K form) at 25°C. Run 1. Load: 190 g. each of sucrose and glucose in 975 ml. solution. Effluent rate: 1 ml./sq. cm. min. Run 2. Load: 200 g. each of sucrose and glucose in 975 ml. solution. Effluent rate: 1/3 ml./sq. cm. min.

at room temperature. The results of this experiment are shown in Table 2.1 and graphed in Figure 2. The curves for ash are omitted in this and other graphs since the ash values correlate very closely with the chloride values. There is only a slight separation of sucrose and non-sugars. The data from a similar run, using 490 g. of 40 purity refiners' cane molasses, show essentially the same results except there is considerable separation of sucrose from reducing sugars (table 2.2).

Runs were made using X-12 and X-4 resins at 90° C. with both beet and cane molasses. The results of the run using beet molasses on X-12 resin in the small column are presented in table 3.1 and Figure 3. X-12

Table 2.1--Purification of beet molasses at 25° C. using Dowex 50 W(K) X-12

Fraction	Percent of total						Purity
	Sucrose	Non-sucrose solids	Nitrogen	Amino nitrogen	Ash	Chloride	
8	2.6	3.3	4.7	2.0	3.3	2.2	61.5
9	16.9	15.9	10.6	9.7	18.8	15.5	67.8
10	24.6	21.8	15.7	13.6	26.8	27.2	69.1
11	23.0	21.8	20.4	17.7	22.8	26.2	67.6
12	16.7	18.5	20.2	18.8	16.0	18.1	64.2
13	9.8	11.4	15.2	16.9	9.1	8.3	63.1
14	4.0	4.9	8.2	11.9	2.6	2.1	61.8
15	1.6	1.9	3.7	6.9	.5	.3	62.6
16	.6	.5	1.3	2.5	.0	.0	69.9
Total g.	262.0	132.3	7.9	1.2	56.4	8.4	

Table 2.2--Purification of cane molasses at 25° C. with Dowex 50 W(K) X-12

Fraction	Percent of total					Purity
	Sucrose	Reducing sugars	Solids less sucrose and reducing sugars	Nitrogen	Chloride	
8	2.9	0.6	6.0	6.7	2.1	35.1
9	17.2	3.4	20.6	18.7	17.3	46.9
10	22.2	8.5	22.2	19.3	25.3	47.8
11	19.1	13.4	20.7	16.7	23.4	42.1
12	18.1	17.1	13.5	14.0	18.7	44.3
13	11.7	19.0	9.9	10.0	11.6	35.9
14	5.7	17.0	4.5	5.3	1.4	27.2
15	1.7	11.3	1.3	3.3	.1	16.7
16	.8	6.3	.7	3.3	-	13.8
17	.5	3.5	.6	2.7	-	16.2
Total g.	148.7	98.9	124.7	2.4	9.5	

separates beet molasses components slightly better at 90° C. than at room temperature but not sufficiently better to be industrially significant. The higher temperature slightly increases the maximum concentration of the individual components, but there is little other advantage. Table 3.2 presents a similar run using 141 g. of 40 purity cane molasses. The results for cane molasses is also poor except for the separation of reducing sugars from sucrose.

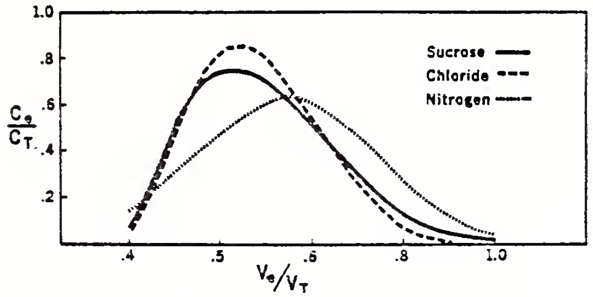

Figure 2.--Beet molasses purification using Dowex 50W (12 percent DVB, 50-100 mesh, K form) at 25° C. Load: 480 g. 65 purity beet molasses in 975 ml. solution.

The most successful separations were made with X-4 resin at 90° C. Runs were

Table 3.1--Purification of beet molasses at 90 C. using Dowex 50 W(K) X-12

Fraction	Percent of total							Purity
	Chloride	Sucrose	Non-Sucrose solids	Nitrogen	Ash	Amino nitrogen	Color	
7	0.2	-	0.2	0.2				
8	6.0	3.9	.5	3.7	5.6	3.1	0.6	-
9	19.0	14.8	16.2	10.6	16.8	8.9	13.0	64.8
10	27.3	23.9	23.8	16.7	25.9	12.5	24.5	66.9
11	30.2	28.2	26.3	21.1	30.5	15.6	30.0	68.4
12	17.4	23.3	23.2	26.5	20.5	21.1	19.3	67.0
13	-	4.5	7.7	15.3	.7	20.6	9.1	53.8
14	-	1.0	1.7	4.1	.1	12.7	2.0	55.6
15	-	.5	.4	1.9	-	5.5	.9	-
16	-	-	-	-	-	-	.6	-
Total g.	2.3	75.4	37.4	2.3	15.2	.4	50.7[1/]	

[1/] Arbitrary color units at 425 mμ.

Table 3.2--Purification of cane molasses at 90°C. using Dowex 50 W(K) X-12

Fraction	Percent of total						Purity
	Sucrose	Reducing sugars	Solids less sucrose and reducing sugars	Chloride	Nitrogen	Color	
7	0.0	0.0	0.2	-	0.4	0.2	0.0
8	4.4	1.0	9.7	7.1	12.1	15.9	31.6
9	19.1	2.4	22.4	23.5	22.5	27.8	46.5
10	26.4	7.1	26.3	29.2	25.0	29.3	47.8
11	27.9	18.5	23.8	28.4	21.4	20.3	44.5
12	18.0	32.2	12.7	11.7	10.7	5.4	34.1
13	3.4	29.7	3.2	.1	4.2	.5	12.3
14	.7	7.9	1.2	-	2.3	.3	8.8
15	.1	1.4	.5	-	1.6	.3	7.7
Total g.	42.7	31.0	38.5	2.8	.6	327.6[1]	

[1] Arbitrary color units at 425 mμ.

made with diluted beet or cane molasses using the small column. The analytical results for the run made with beet molasses are presented in table 4 and Figure 4. Nearly 50 percent of the sucrose was eluted at 80 purity or higher. Most impurities are eluted before the maximum sucrose concentration, but some appear later. Impurities eluted later are principally amino acids and other nitrogen containing compounds.

This experiment was repeated using cane molasses, (table 5 and Figure 5). More than 65 percent of the sucrose was eluted at 68

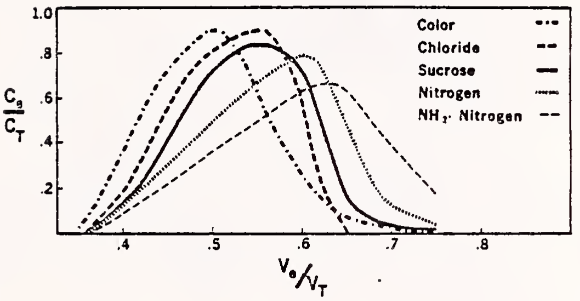

Figure 3.--Beet molasses purification using Dowex 50W (12 percent DVB, 50-100 mesh K form) at 90° C. Load: 133 g. 65 purity beet molasses in 270 ml. solution.

Table 4.--Purification of beet molasses at 90° C. using Dowex 50 W(K) X-4

Fraction	Percent of total							Purity
	Sucrose	Chloride	Non-Sucrose solids	Nitrogen	Amino nitrogen	Color	Ash	
7	-	0.2	0.3	0.4	0.1	1.7	0.3	-
8	-	1.7	3.0	2.3	1.6	10.1	2.9	-
9	-	5.9	6.9	4.8	4.3	15.0	7.3	-
10	0.6	11.6	10.7	7.4	7.2	19.2	12.6	9.1
11	3.9	16.8	14.3	10.1	9.8	21.7	16.9	34.3
12	12.1	22.1	17.0	11.7	12.1	15.5	21.1	57.4
13	21.9	29.7	19.6	13.2	12.7	7.0	25.8	68.0
14	29.5	12.0	11.7	14.7	19.0	6.4	11.6	82.7
15	19.6	-	8.4	16.9	11.8	2.4	1.1	81.5
16	12.1	-	6.8	14.5	12.4	.8	.3	77.0
17	.2	-	1.2	3.8	9.2	.3	.1	27.6
Total g.	76.2	2.3	40.2	2.2	.3	48.7[1]	15.0	

[1] Arbitrary color units at 425 mμ.

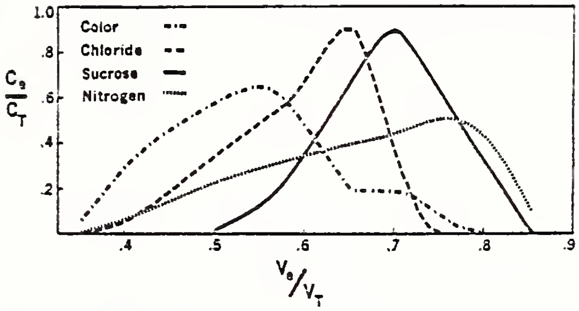

Figure 4.--Beet molasses purification using Dowex 50W (4 percent DVB, 50-100 mesh, K form) at 90° C. Load: 133 g. 65 purity beet molasses in 270 ml. solution.

purity or higher. This purity is a considerable improvement over that obtained using X-12 at 90° C., where the best fraction had a 48 purity. It is likely that the substances shown in the early portions of the reducing sugar curve are not reducing sugars but higher molecular weight compounds or salts that exhibit reducing properties. Comparison of the color curves for the beet and cane molasses runs shows that both depart considerably from a smooth curve at fractions 12 to 14. This anomaly is due to some colored substance that is excluded less from the resin than the majority of colored compounds.

The difference in the nature of the nitrogen compounds present in beet molasses from those in cane molasses is shown by a comparison of the separation of beet and cane components on Dowex 50W (4 percent DVB, 50 to 100 mesh, K form) at 90° C. Most of the cane molasses nitrogen compounds are eluted in the first half of the run, but most beet molasses nitrogen compounds are eluted in the later half. The late elution of beet molasses nitrogen compounds is primarily due to the presence of relatively larger amounts of amino acids and betaine. The separation of reducing sugars from other material is quite good. More than 70 percent of the reducing sugars can be obtained at 78 purity compared with an original 27 purity. More than half of the non-reducing fraction is sucrose so that the total sugar purity (no. 15-18) is 90.

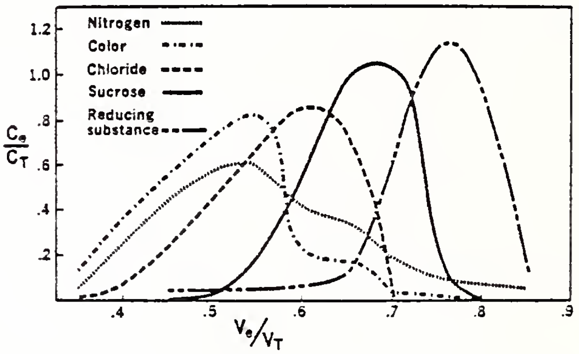

Figure 5.--Cane molasses purification using Dowex 50W (4 percent DVB, 50-100 mesh, K form) at 90° C. Load: 141 g. 40 purity cane molasses in 270 ml. solution.

Table 5.--Purification of cane molasses at 90° C. using Dowex 50 W(K) X-4

Fraction	Percent of total							Purity
	Sucrose	Reducing sugars	Solids less sucrose and reducing sugars	Chloride	Nitrogen	Ash	Color	
7	-	-	1.3	0.4	1.6	0.7	3.8	-
8	-	-	6.8	2.4	8.8	4.2	12.3	-
9	0.1	0.8	11.5	7.6	14.5	10.3	18.4	1.4
10	1.0	1.1	16.2	15.1	17.6	16.7	21.8	6.6
11	6.7	1.5	18.9	22.1	17.9	22.3	27.2	29.3
12	17.8	1.5	19.0	28.4	13.8	23.9	7.1	52.3
13	32.9	3.8	16.2	23.7	11.6	20.6	6.0	68.1
14	34.0	19.8	2.9	.1	6.3	.6	1.0	68.6
15	7.5	36.9	2.9	-	3.5	.3	.8	21.7
16	.1	30.1	2.4	-	2.5	.2	.6	.3
17	-	4.3	1.1	-	1.9	.2	.5	-
18	-	-	.8	-	-	-	.4	-
Total g.	45.6	30.5	36.3	2.8	0.6	15.9	323.0[1]	

[1] Arbitrary color units at 425 mμ.

Conclusions

Examination of the results of these experiments indicates that X-4 resin at 90° C. gives the best purification. Under these conditions 50 percent of the sucrose in beet molasses was separated at 80 purity or higher. On a commercial scale these fractions could be returned to intermediate pans using liquors of this purity for crystallization and sucrose recovery. Fraction 16, 77 purity, containing 12 percent of the sugar could be returned to the low raw pans but fractions 12 to 13 containing 34 percent of the sugar are of such low purity that under present practice they should not be returned to the sugar end of the factory. Most of the sugar in these fractions may be recovered by using them to dilute fresh molasses for the next column load. The impurities returned to the sugar end would of course form some molasses and later be recycled through the column. Using ion exclusion it would not be necessary to obtain only highly purified fractions. High purity fractions would be returned to the sugar end of the factory for concentration and crystallization, fractions high in salts and low in sucrose would be discarded, and fractions intermediate in purity would be used to dilute fresh molasses and recycled on the column.

The results of these experiments show that static ion exclusion columns of Dowex 50W (4 percent DVB, 50 to 100 mesh, K) can be used to recover sucrose from beet and cane molasses and invert from cane molasses.

Operation of the columns at 90° C. gives the best overall separation of molasses constituents, the highest concentration of solids, and lowers the possibility of fermentation taking place during purification.

Acknowledgment

I wish to thank the following people for analytical determinations: Mr. E. F. Potter (reducing sugars and sucrose) and Mr. H. Wright (solids, ash, total nitrogen, and amino nitrogen). I am indebted to the Spreckels Sugar Co. and to California and Hawaiian Sugar Refining Corp. for samples of beet and cane refiners molasses.

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DISCUSSION

S. Borodkin (American): Dr. Stark, when you say you recycle the partially separated molasses, do you mean over the same resin column or a fresh resin column?

J. B. Stark (WURDD): The same resin column. You can do this in two ways. You can either save the cuts if you are operating a plant and run them over, or as far as I can see, it makes little difference if you just recycle them almost immediately.

If you have an in-line refractometer, the fraction up to an RDS of 11 or so will have practically no sugar. So this is thrown away. Then we can take another cut up to about 20 RDS which we use to dilute fresh molasses to bring it down to about 40 Brix.

The cut from about 20 Brix through the maximum of around 28 and down on the other side is taken for the product. This fraction is either a product or it can be recycled with other products of a similar nature.

This whole process to the end of the product fraction is about eight tenths of the bed volume. At five tenths of the bed volume we start the next load. Before the first one is finished, the next one is going on. In cane, of course, we would also have another major fraction at the end consisting of your reducing sugars, and it takes about a tenth of a bed volume longer for these materials to come off than it does in the case of beet molasses, so you'd have to add your new load on at about 0.55 of the bed volume.

And, also, something else you probably would have to do with cane would be a sodium calcium exchange. There is a lot more calcium in cane molasses than there is in beet. The resin in the calcium form will not function as successfully as it will in the sodium or potassium form.

T. M. Rinehart (Atlas): How many bed volumes of water did you have to use?

J. B. Stark: Well, let's see, we put on a load about every half bed volume. This new load is 0.15 of a bed volume. So it would be 0.35, thirty-five hundredths of the bed volume would be eluant; that is the water fraction. And I think you can squeeze this down to 0.45 for your total distance between the loads. That would decrease the amount of water around 20 percent.

You have the problem of waste also. What are you going to do to these wastes? Do you have to evaporate them? Do you run them into the river?

You are going to lose 3, 4, or 5 percent sugar, depending on how far you want to make your cuts.

N. H. Smith (C&H): Have you tried the ionic retardation methods? Do they work?

J. B. Stark: I think they probably would, but there is one difficulty. I think you could separate the impurities, a good many of the ionic impurities, but the color is going to go along in the sucrose. Ion exclusion has the advantage of removing a lot of the color. But in ion retardation, the sucrose is on the outside of the resin bead, and so is the color. They are going to come through together.

I might also add that ion exclusion has the advantage over ion exchange of being able to get rid of some of the nonionic materials such as reducing sugars and raffinose.

E. E. Coll (SURDD): Do you have any information on the effective cycle life of the resins?

J. B. Stark: No. I think that it's probably quite long. This is one of the advantages that you have over ion exchange. You are using a cation exchanger, which is about the most stable of the resins. It's going to last a lot longer certainly than the anion exchanger in your ion exchange system.

E. E. Coll: And what are your flow rates?

J. B. Stark: Right now most of them are milliliter per square centimeter. This is equivalent to a quarter of a gallon per square foot per minute. We were going higher, but with this small column, it's difficult.

We did make one run on sucrose and salt at 2 1/2 gallons per square foot per minute, and there wasn't much difference, but you are just too busy taking cuts and trying to find out what is going on to handle this with a small column. Now, we are having a 10-foot column constructed, 6 inches in diameter. There will be a lot more time between fractions, even at the same flow rate, so we will try much higher rates.

E. E. Coll: Is the relative shape of your curves the same on a recycle?

J. B. Stark: Relatively speaking, yes, but not quite the same, because your impurities have changed. The material you are putting on is not quite the same. For instance, if you have more salt, this drives the concentration of the sucrose up to a higher level, which is beneficial, but it is also detrimental because you will have a little more salt in your product.

E. E. Coll: I notice that the plateau on the decreasing side of the color curve coincides with the maximum chloride concentration. Did you investigate that?

J. B. Stark: No, we have not looked into this, but it's a very distinct band on the column.

I would like to have Mr. Mountfort say a few words. He has some experience with this matter of ion exclusion.

C. B. Mountfort (Colonial Sugar Refinery, Australia): We haven't tried using molasses, I think the problems with cane molasses would be the relatively high concentrations of calcium and magnesium which you would have to remove first. I am sure you would get into trouble with those.

On refinery affination syrups you get quite good results. You can raise the purity from 90 up to about 97, eliminating about 95 percent of the ash and about 80 percent of the color and perhaps about 60 percent of the unknowns. You have a good flow rate. I think you can probably get to at least 0.6 or 0.7 gallon per minute per square foot.

J. B. Stark: You are now considering factory scale operations of this process, are you not?

C. B. Mountfort: Yes, I have an 8-foot diameter column just about finished now, but I don't have any results yet.

SALT CONCENTRATIONS IN A SUCROSE GRADIENT

by
K. M. Onna
Experiment Station
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Concentration gradients were developed by crystallizing and dissolving sucrose crystals in the presence of various salts, and by mixing two solutions of different sucrose concentrations. Analytical data for sodium chloride, potassium chloride, calcium chloride, calcium sulfate, and calcium monobasic phosphate indicated salt concentration shifts in the sucrose gradient. Evidence shows that the altered salt concentrations may influence crystallization and inclusion. This influence differs from the influence of salt concentrations measured in the bulk liquor. Findings suggest a highly circulating system as a basis for crystallization rate and inclusion studies.

Introduction

In a previous study (1) of the relation between nonsucrose constituents and crystal size in raw sugar it was demonstrated that the impurities were retained predominately by inclusion within the crystal rather than by adsorption on the surface. These studies were based on the measurement of color, specific conductance, and 85 percent methanol-insoluble substances. Considerable attention was focused on the entrapment of the mother liquor as a type of inclusion. The idea that impurity concentration might be a function of internal water also developed.

Some work at this Station has shown that impurity balance does not necessarily follow water balance. If the color per unit brix of the crystal is compared to that of its corresponding syrup, the value for the crystal may be 10 to 33 percent of the value for the syrup. This appears to be a high value in terms of the usual concept of crystallization as a means of purification. Crystal color is due to both light absorption and light scattering. In this connection it has been reported that light scattering appears too high to be attributed to the inclusion of the bulk-analyzed mother liquor (2).

In a study of salt inclusion, some results were obtained where the salt per unit internal water approached the salt per unit water for the corresponding mother liquor; yet, even in these cases, small differences seem to persist (3).

The solubility of sucrose in the presence of salts has been extensively studied (4, 5, 6, 7). With sodium chloride and potassium chloride it has been shown that the amount of sucrose which will dissolve in 1 g. water at a given temperature decreases and then increases as the salt concentration rises.

If the crystallization rate curve at 30.6° C. of Moritsugu (8) is superimposed over the solubility diagram at 30.0° C. of Schukow (4), it is readily seen that maximum rate does not coincide with minimum solubility, hence, neither with maximum supersaturation (Figure 1). The direction in which the rate curve seems to have been transposed and the somewhat higher ratio of salt-to-water for the crystal than for the corresponding mother liquor, as mentioned earlier, point to the possibility that during crystallization the effective salt concentration might have shifted.

Experimental Procedures

The basis for a study of the salt-to-water ratio in a sucrose gradient may be illustrated in a simple manner. In a mother liquor consisting of sucrose, an impurity, and water in the proportion 4:4:1, purity will be 50 percent and the ratio of impurity-to-water 4:1. If two parts of sucrose crystallize out without evaporation of water, the proportion of sucrose, impurity, and water will now be 2:4:1. Purity will have dropped to 33 percent, but the ratio of impurity-to-water will remain 4:1. As crystallization proceeds, the supersaturation of the mother liquor drops in the vicinity of the crystal surface. The ratio of impurity-to-water in this region may now change.

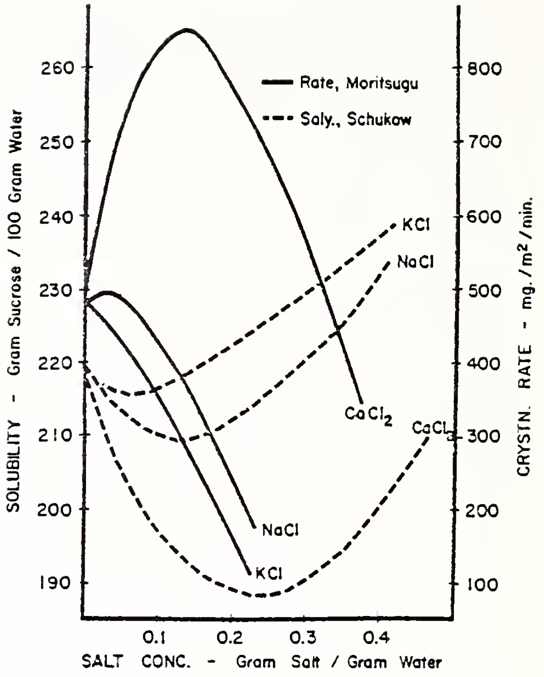

Figure 1.--Sucrose crystallization rate and solubility in presence of salt.

Sucrose concentration gradient column. -- A concentration gradient column (Figure 2)

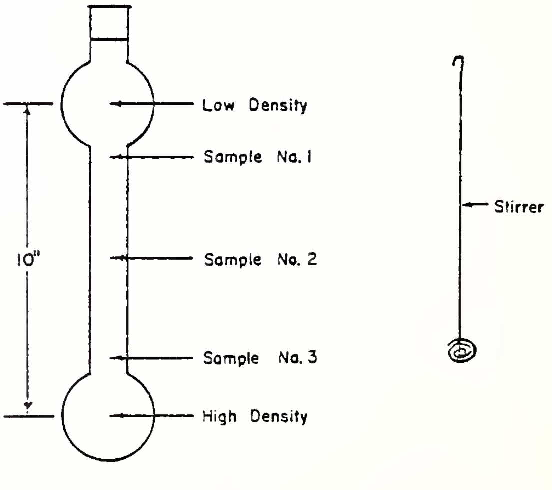

Figure 2.--Sucrose concentration gradient column.

was constructed. The tube was half filled with an aqueous solution high in sucrose. A solution low in sucrose but with the same ratio of salt-to-water was introduced above the high sucrose solution. The two solutions were mixed for 1 minute by steadily increasing the strokes of the stirrer, beginning at the midpoint. This resulted in a sucrose gradient along the length of the column. After 16 to 20 hours at 30° C., samples were taken at five different points. Moisture was determined refractometrically. Salts were determined by specific conductance of aqueous dilutions which were referred to specific conductances of known salt concentrations containing the same amount of sucrose (less than 1.5 percent) as in the sample. Results are shown in table 1.

Table 1.--Salt concentration in a sucrose concentration gradient column at 30° C.

Salt	mg. Salt/g. Water[1]				
	Low Density	Sample #1	Sample #2	Sample #3	High Density
KCl	1.02	1.06	1.08	1.04	1.05
	(0.3)	(9)	(28)	(57)	(65)
$CaCl_2$	9.16	9.12	9.09	9.22	9.20
	(2)	(6)	(34)	(46)	(64)

[1] Values in parentheses represent percent sucrose in solution.

Concentration gradient by crystallization of sucrose crystals.--Sucrose crystals (0.5 x 0.6 x 0.8 mm.) were bonded (Figure 3a) to the inner wall of a 500 ml. Erlenmeyer flask with organic solvent-soluble water-insoluble bonding agents: Varniton Label Varnish (Varniton Co., Burbank, Calif.) and rubber cement (Carters Ink, Cambridge, Mass.). These materials provided a loose bonding but, as the crystals grew in a supersaturated solution, a firm bonding resulted. Undesired crystals at the bottom were dissolved in water. The flask was rinsed with dimethylformamide, methanol, and isopropanol, and was then dried.

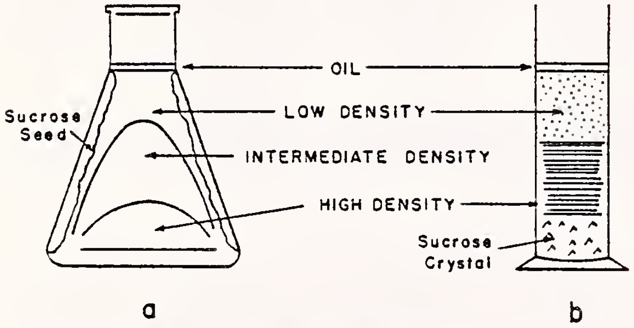

Figure 3.--Development of sucrose gradient in a salt solution: a, Crystallization of sucrose, b, dissolution of sucrose.

A salt solution supersaturated with sucrose was introduced and topped with paraffin oil. As crystallization of sucrose proceeded, the low-density layer accumulated at the top. Analyses of the top, central, and bottom layers were carried out (tables 2, 3, and 4).

Concentration gradient by solution of sucrose crystals.--Sucrose crystals were dissolved in a salt solution (Figure 3b). As the

Table 2.--Salt in sucrose concentration gradient by crystallization at 30° C.

	mg. Salt/g. Water[1]		
	Low Density	Int. Density	High Density
$CaSO_4.2H_2O$[2]	1.31	1.21	1.14
	(68.1)	(70.6)	(74.5)
" " " " "	1.17	1.11	1.00
	(67.9)	(69.4)	(71.8)
" " " " "	1.20	1.20	1.08
	(68.7)	(70.0)	(72.9)
NaCl	1.11	1.01	0.97
	(69.9)	(71.8)	(74.1)
KCl	1.06	1.06	1.02
	(67.9)	(70.8)	(75.0)
$CaCl_2$	1.03	1.01	0.87
	(69.8)	(71.3)	(74.5)
$Ca(H_2PO_4)_2.-H_2O$	1.03	1.08	0.98
	(67.1)	(71.7)	(73.8)

[1] Values in parentheses represent percent sucrose.

[2] Crystallization time with $CaSO_4.2H_2O$ - 48 hours; other salts - 24 hours.

crystals dissolved, a syrup layer developed. Samples were taken on both sides and close to the boundary between the syrup and salt solution table 5.

Table 3. --Salts in sucrose concentration gradient by crystallization at 40° C. for 24 hours

	mg. Salt/g. Water[1]	
	Low Density	High Density
NaCl	1.02 (68.0)	1.00 (74.6)
KCl	1.04 (70.4)	0.94 (74.2)
$CaCl_2$	1.04 (70.4)	0.96 (74.4)
$CaSO_4 . 2H_2O$	1.14 (70.8)	1.03 (72.3)
$Ca(H_2PO_4)_2 . H_2O$	3.52 (72.3)	3.33 (75.0)

[1] Values in parentheses represent percent sucrose.

Discussion

Table 1 indicates that the formation of a sucrose gradient does not mean the occurrence of a measurable salt shift. If ionic mobilities are considered, then a considerably longer time than allotted may be required for the salt concentration to shift over a 10-inch distance. The disadvantage of an extended time is that sucrose also moves in the direction of decreased concentration; hence the gradient tends to be destroyed.

Table 4. --Salts in sucrose concentration gradient by crystallization at 50° C. for 16 hours

Salt	mg. Salt/g. Water[1]		
	Low Density	Int. Density	High Density
NaCl	1.15 (72.1)	1.15 (72.5)	1.04 (74.3)
KCl	1.16 (72.1)	1.11 (72.5)	1.06 (73.9)
$CaCl_2$	0.75[2] (72.0)	0.75 (72.1)	0.94 (74.4)
Do.	1.26 (73.1)	1.21 (73.6)	1.11 (74.6)
Do.	1.29 (73.2)	1.19 (74.0)	1.13 (75.6)
Do.	1.23[2] (72.6)	1.26 (73.0)	1.28 (75.6)
Do.	0.26[3] (73.1)	0.25 (74.0)	0.24 (75.1)
$CaSO_4 . 2H_2O$	0.83[2] (72.9)	0.84 (73.3)	1.03 (76.7)
$Ca(H_2PO_4)_2 . H_2O$	1.04 (76.7)	0.95 (77.0)	0.95 (77.4)

[1] Values in parentheses represent percent sucrose.
[2] Inconsistent trend.
[3] Reduced salt concentration and moderate heating to prepare syrup.

Table 5. --Salts in sucrose concentration gradient by solution at 30° C. for 16 hours

	mg. Salt/g. Water	
	KCl	$CaCl_2$
Original solution	0.51	0.45
Low density solution (3 percent sucrose)	.50	.47
High density solution (57 percent sucrose)	.51	.40

If a thin layer of a low density sucrose solution is passed through a second solution of higher density, the time required for migration can be reduced. The problem with this approach is that the thin layer is subjected to destruction and may not survive long movements. If, however, the thin layer can be protected in some manner and allowed to accumulate, sufficient samples can be obtained for analyses.

The formation of such a layer is accomplished by a simple crystallization technique. The flask wall is used to retain the crystals. The thin layer developed by this method regenerates itself by continuous loss of sucrose molecules to the seed crystals on the wall. Because of its decreased density, the low density layer accumulates at the top of the flask.

Tables 2 and 3 indicate that at 30° and 40° C. the amount of sodium chloride, potassium chloride, calcium chloride, calcium sulfate, and calcium monobasic phosphate per unit water tends to be higher in the region of high sucrose concentration. At 50° C. some change from this pattern seems to occur with calcium chloride and calcium sulfate (table 4). The reason for this is not clear, but may involve the heat of crystallization.

Table 5 presents the results obtained from a gradient by solution of sucrose crystals. The values for potassium chloride and calcium chloride per unit water in the syrup and adjacent salt solution suggest that these two salts might not redistribute themselves to the same degree.

The failure of maximum crystallization rate to coincide with maximum supersaturation may now be explored.

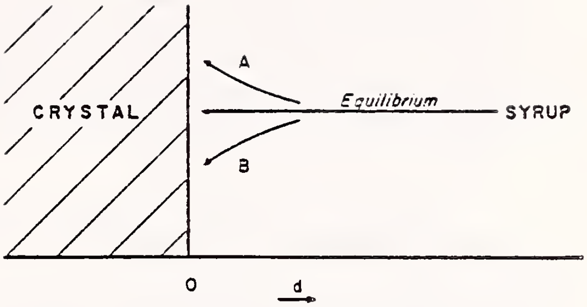

Figure 4.--Impurity distribution near crystal surface.

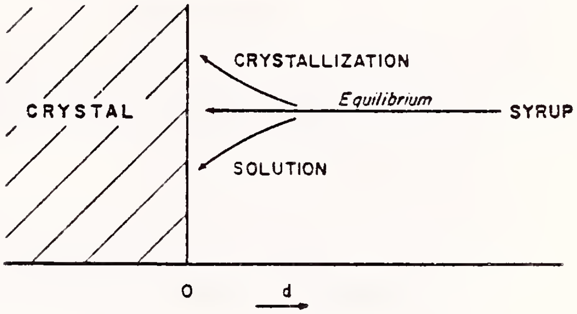

Figure 5.--Saturation temperature near crystal surface.

Measurement of salt concentrations in a sucrose gradient by crystallization leads to a distribution pattern shown in Figure 4. The variation in impurity concentration means the existence of the saturation temperature profile (Figure 5); A and B represent changes in saturation temperatures, depending on whether impurity increases or decreases sucrose solubility. In the absence of a salt there is a reduction in supersaturation around a growing crystal. If, however, a salt is present and a shift in its concentration occurs, the supersaturation curve may shift to a value higher or lower than the value in the absence of the salt (Figure 6). In a poorly circulating system with a decreasing curve, the rate of sucrose crystallization may become very low since the gradient shell persists. For this reason Smythe (9) has suggested a highly circulating system as a basis for studying influences of substances affecting rate of crystallization.

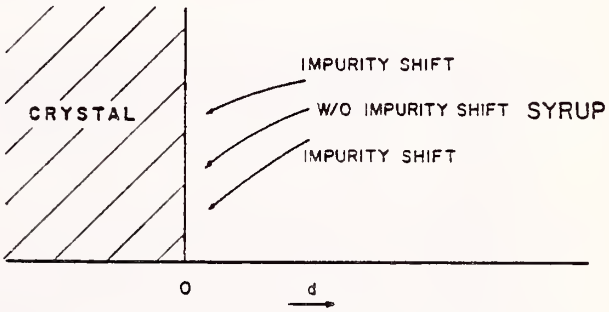

Figure 6.--Supersaturation near crystal surface.

Conclusions

The salt concentration is affected by the concentration gradient that surrounds a growing or dissolving sucrose crystal.

In the concentration range studied and at 30° and 40° C., the amount of sodium chloride, potassium chloride, calcium chloride, calcium sulfate, and calcium monobasic phosphate tends to be higher in the region of low sucrose concentration than in the region of high sucrose concentration.

Salt shifts may exert their own influence on crystallization and inclusion.

Crystallization under nonequilibrium conditions and highly circulating systems may be valuable adjuncts in inclusion and rate studies.

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THE QUANTITATIVE DETERMINATION OF ORGANIC ACIDS BY ION EXCHANGE CHROMATOGRAPHY AND COULOMETRIC TITRATION

by
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(Presented by S. E. Borodkin)

A method for the separation and quantitative determination of organic acids is presented. The acids are first resolved on an anionic exchange resin in the formate form using a nonlinear gradient of increasing formic acid concentration as eluant. The column effluent is collected in an automatic fraction collector. The formic acid is removed from the individual fractions by the addition of chloroform and the subsequent volatilization of the low boiling chloroform-formic acid azeotrope. The residue is then taken up in 70 percent isopropanol and titrated coulometrically with automatic endpoint detection.

Thin layer chromatograms are normally run on each tube as a means of identifying the acid(s) present and determining the purity of the fraction. This technique, although presented here as an auxiliary method, may be used independently.

Introduction

This project had as its aim the development of a single chromatographic technique, of high resolution, suitable for the determination of both solid and volatile acids and which can be used for the analysis of all refinery materials with purities ranging from blackstrap molasses to granulated sugar.

A review of the literature revealed that the determination of organic acids in sugar products has been the subject of several recent publications.

The methods used fall into four categories:

1. Partition Chromatography on Silical Gel Columns (1, 2).
2. Ion Exchange Chromatography (3).
3. Gas Chromatography (4, 5).
4. Paper Chromatography and Electrophoresis (6, 7, 8, 9).

At the 1959 Technical Session on Bone Char, during the discussion following the presentation by Roberts and Martin on organic nonsugars, Gross (Tate and Lyle) and Martin (Southern Regional) concluded that the procedure presented for the determination of carboxylic acids in cane juices (1) was not suited for the analysis of materials beyond the washed sugar stage.

Pretreatment with anion exchange resins for the purpose of concentrating the acids in the higher purity refinery products, preliminary to partition chromatography, was studied. We were, however, so impressed with the inherent advantages of ion exchange, that we attempted to use it for the chromatographic separation of the acids.

Ion exchange chromatography is superior to silica gel methods in the following respects:

1. Anion Exchange resins have little affinity for positively charged and neutral substances so that cations, carbohydrates, etc., which are not adsorbed and could possibly interfere with the elution and titration of the acids, are washed free from the resin.

2. Partition chromatography precludes the addition of aqueous samples to the silicic acid column because excess water will alter the equilibrium between the aqueous stationary phase and the mobile organic phase. Therefore, either a drying or an extraction technique is necessary to remove the water from dilute samples. Some acids might be lost in this step. Aqueous solutions are used generally with ion exchange resins.

3. The much greater capacity of ion exchange resins permit the use of smaller columns, shorter eluting times, and higher acid concentrations.

4. Elution is accomplished with a gradient of increasing concentrations of formic acid. The formic acid is removed by azeotropic distillation with chloroform (10) leaving the acid in a pure form for easy identification, if necessary.

Analytical Method

It seemed fairly obvious that the biggest problem would be the development of a method with sufficient sensitivity to determine the small quantities of acid present in refined products. Several alternatives, which have been tried by various workers in the field, are (1) use of a large sample size, (2) use of dilute base (0.005N NaOH) as titrant, and (3) use of an extractant such as ether or steam distillation of volatile acids. The extraction techniques are likely to be nonquantitative in separation of acids, while steam distillation has the additional problem of possibly creating more acids.

Instead of pursuing these techniques, we have decided to use a coulometric titration for the quantitative determination of the acids obtained from the column effluent.

The successful application of this technique to the analysis of the ion exchange effluents has truly transformed column chromatography of organic acids into a microtechnique. Typically, a 1-g. sample is sufficient for the analysis of a raw sugar and 10 g. are adequate for the analysis of a granulated sugar.

The first application of anion exchange chromatography for the separation of acidic material was done in 1950 when W. Cohn (11) used this technique for the isolation of nucleic acids. He found that by decreasing the pH in a stepwise fashion, he could elute the nucleotides in a predictable sequence, dependent upon their degree of association. Utilizing formic acid instead of the original hydrochloric acid eluant, he found that he was able to control the pH more accurately, thereby obtaining more reproducible results.

Busch, Hurlbert, and Potter (12) utilized a similar technique for the separation of the Krebs cycle acids. A gradient elution device was employed to obtain a gradual increase in the concentration of formic acid. Evaporation of the formic acid + water was effected by the use of a vacuum desiccator with an infrared heater. The recoveries were good except for the cis-aconitic and alpha-ketoglutaric acids which were partially decomposed by the method of drying.

Owens, Goodban, and Stark (3) separated the organic acids in sugar beets on an anion exchange resin. The resin was added in two parts. The bottom portion was equilibrated with hydrochloric acid or oxalic acid to a pH of 2.0 to 2.5, while the top portion was equilibrated to a pH of 2.5 to 4.2 with formic acid. The acids contained in the effluent were not titrated acidimetrically but were quantitatively determined by tests specific for each acid.

Palmer (13) in his investigations of the organic acid content of the tobacco plant used either formic or acetic acid as the eluting agent. He evaporated off the eluant in the test tubes with a stream of air flowing through glass capillaries. During the evaporation, the temperature was maintained at 46° to 48° C. in a water bath. The recoveries of cis-aconitic and alpha ketoglutaric were considerably improved over those reported by Busch and others (12).

Sullivan, Voelker, and Stahl (14) used this ion exchange method with a formic acid gradient for the separation of the organic acids in vanilla. They introduced, however, an ingenious innovation for the removal of formic acid. This was based on the fact that formic acid and chloroform forms a low boiling azeotrope that can be distilled off leaving the remaining organic acids intact. Recoveries, in the range of 97 percent to 103 percent, were reported on known acid mixtures when this modification was applied to the formate column method used by Palmer.

Modifications of the basic anion exchange method have been made by various workers in the field, and it has been applied successfully to the determination of acids in various fruits (15, 16) and potatoes (17).

Coulometric Titration

A coulometric titration can be described as the titration of a substance using an electric current for the titrant instead of a standard solution. This is accomplished by generating a reagent electrolytically which reacts stoichiometrically with the material in solution. The quantity of the substance titrated is calculated directly from Faraday's Law, which states that to produce one equivalent of chemical change requires 96,493 coulombs (a coloumb is the quantity of electricity required for the flow of one ampere for one second). Since we generate with a constant current, the time elapsed is a direct measurement of the coulombs passed.

The extreme sensitivity of the method is due to the fact that very small currents can be generated accurately. The time elapsed is directly proportional to the coulombs and, since time can be measured accurately to 0.1 second or better with an electrical stop-clock, the inherent reproducibility of coulometry is excellent. Furthermore, it eliminates the need for the preparation and standardization of carbonate free reagents and the storage of the dilute alkaline titrant in a carbon dioxide free atmosphere.

The acid (i.e., hydrogen ion, H^+) is titrated either by the electroreduction of the hydrogen ion at a platinum electrode

$$2H^+ + 2e^- \longrightarrow H_2\uparrow \quad (1)$$

or by the reaction with hydroxyl ion generated by the electrodecomposition of water

$$2H_2O + 2e^- \longrightarrow 2OH^- + H_2\uparrow \quad (2)$$

The reaction occurring at an inert platinum anode would be

$$2H_2O - 4e^- \longrightarrow 4H^+ + O_2\uparrow \qquad (3)$$

If the anode is then placed in the same compartment as the cathode, the H^+ thus produced at the anode would be titrated by the cathode according to reaction (1). The resultant effect would be that the titration would continue until all the water was decomposed. To circumvent this, we can separate the anode and cathode from each other with a salt bridge (3 percent KCl in agar) to maintain electrical contact. An alternate technique employed is to use a silver wire for the anode in a dilute KBr or KCl solution. In this way reaction (4) occurs at the anode

$$Ag + Br^- - e^- \longrightarrow AgBr \qquad (4)$$

Reaction (4) seemed preferable because the titration is performed in a single chambered cell without a salt bridge and, therefore, most of the titrations were performed in a cell similar to Figure 1.

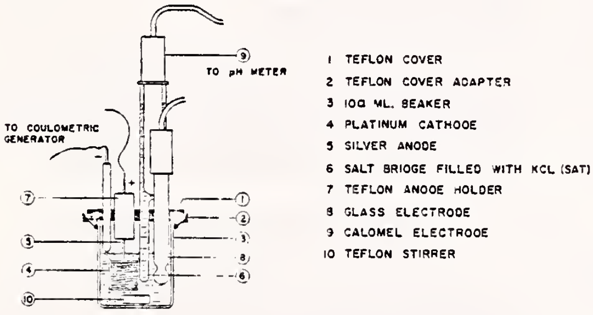

Figure 1.--Cell for coulometric titration of acids using a platinum generator cathode, silver anode and pH electrodes for endpoint detection.

The anode is silver and the cathode is a heavy gauge platinum coil. The titration is performed in 70 percent isopropyl alcohol (18) containing a few drops of potassium bromide. Isopropyl alcohol is used because some of the acids are insoluble in water and also the dissociation constants of the various organic acids are decreased. This has a leveling effect on the acids so that the equivalence point, which occurs at pH 9, is almost exactly the same for all the acids titrated.

Figure 2 is a photograph of the titration system used.

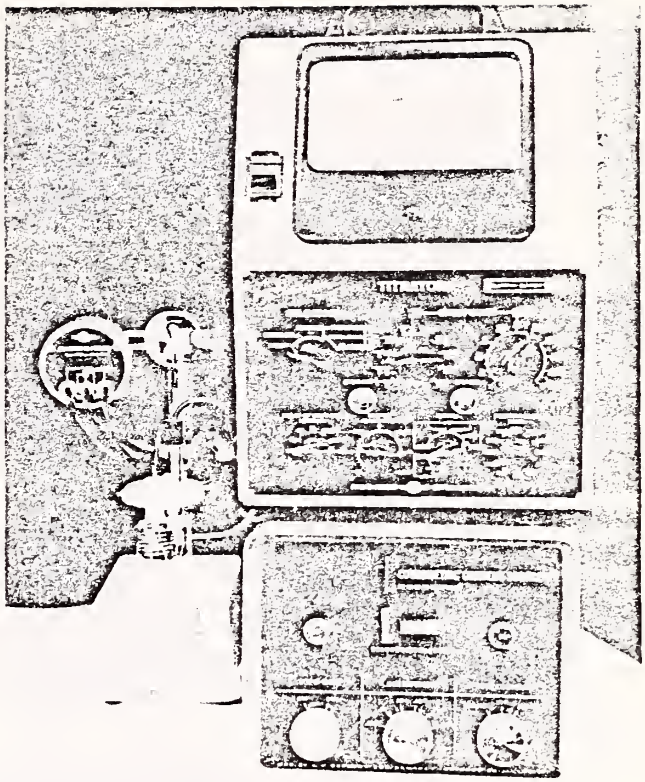

Figure 2.--Apparatus for automatic coulometric titration of acids. The coulometric current source is operated in the remote position and is controlled by the titrator through a relay.

The positive and negative output terminals of the coulometric current source are connected to the silver and platinum electrodes, respectively, via a 2-conductor cable.

The output jacks of the calomel and glass electrodes are joined to the input terminals of an automatic titrator. The coulometric current source is operated in the remote position and is shut off automatically at the preset endpoint, pH 9.0, by the titrator through a magnetic relay.

A titrator with an anticipatory circuit which will shut off prior to the endpoint is necessary because while the generator is in operation, we cannot obtain a true pH reading. The Beckman Model K Autotitrator or the Radiometer Titrator TTT1 possess a controlling device for setting a false endpoint somewhat in advance of the true endpoint. When this pH is reached, a relay is activated which turns off the generator and, since the pH is not yet at the true endpoint, a second relay is actuated and the generator is turned back on. This process continues, converging on the endpoint, until it is reached.

General Procedure

Dowex 1-x8 of 200-400 mesh size is slurried with water and allowed to settle for about 5 minutes. The supernatant suspension of fines is poured off and the process repeated several times until most of the very fine particles are removed.

The resin is then washed repeatedly with 1N sodium formate until the resin is completely in the formate form (the washings give a negative test for chloride). The resin may be then stored in slurry form in an amber bottle until needed.

The chromatographic column is filled to a height of 35 cm. with the resin slurry and washed with about 150 ml. of water. The sugar sample, dissolved in H_2O, is added to the top of the column and washed through with about 150 ml. of water at a flow rate of about 3 ml. a minute.

A 3-chambered Varigrad (19) is used to maintain the desired gradient of formic acid concentration. The first chamber is filled with 250 ml. of water and the second and third chambers are filled with 250 ml. of 6N formic acid in each. The formic acid gradient emerging from the outlet of the first chamber is introduced through an air-tight fitting to the top of the ion exchange column. A metering pump which can operate at pressures up to 20 pounds per sq. inch is desirable. Best results were obtained using the Technicon AutoAnalyzer pump which also has the additional advantage of being able to run several columns simultaneously. Flow rates are maintained at about 1 ml./min.

The eluate is collected in an automatic fraction collector which is normally set to collect 4-5 ml. fractions. In calibrating a column with known acids, or if occasionally more complete separations are required the fraction collector is set to collect 1 ml. fractions.

Figure 3 is a photograph of the chromatographic column and fraction collector.

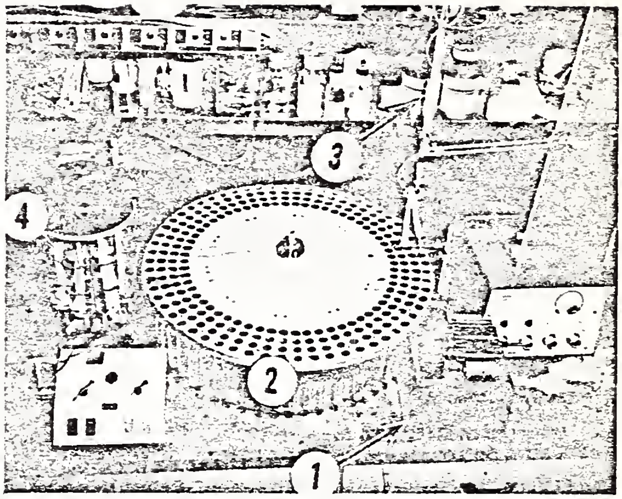

Figure 3.--Apparatus used for the ion-exchange chromatography of organic acids: (1) Multichannel Metering Pump, (2) Fraction Collector, (3) Chromatography Column, and (4) Varigrad.

The test tubes containing the eluate are removed from the fraction collector; 1 ml. of chloroform is added to each and placed in a rack in a water bath at 50° C. A glass manifold fitted with capillary tubes is suspended above the test tubes in the rack. Clean, dry air is blown over the surface of the liquid in the tubes so that the removal of the water and chloroform azeotropes is effected as rapidly as possible to obviate the loss of some of the more volatile acids.

When evaporation is complete, the residue in the test tube is taken up in 70-percent isopropyl alcohol, transferred to the titration cell, and titrated coulometrically.

Thin Layer Chromatography

The need for a rapid method that could separate out small amounts of organic acids became apparent early in our investigation. Thin layer chromatography (TLC), a relatively new technique, seemed especially attractive for our purpose (20).

TLC is often referred to as "open column" chromatography, where the adsorbent is deposited in a thin layer on a glass support instead of the customary column. In operation it is very similar to paper chromatography only much faster. Unlike paper chromatography we are not restricted to one type of media, but many types of adsorbents may be spread on the glass plates. In addition, the highly inert adsorbents and glass plates can withstand corrosive spray reagents that cannot be used on paper. The chief advantages for us, however, are its great speed (15 min. for separation of the liquid acids), and sensitivity. Since only about 10 μg. of acid are required, we can use this technique directly on the column effluent by withdrawing a few microliters from the test tube. This insignificant amount has a negligible effect on the titrations.

Unfortunately, to date, we have not been able to find a single solvent system capable of separating all of the liquid and solid acids with TLC. We are experimenting with simple derivatives, e.g., amine salts, methyl esters, acid halides, and others, in an attempt to solve this problem. Additionally, we hope the use of derivatives will permit the employment of more sensitive sprays for the detection of the acids. Presently, on systems low in acid content, the only practical means of identification possible is by the comparison of the peak volume of the effluent acids with those of known acids.

Separation of Liquid Organic Acids

Layers of silica gel 0.25 mm. thick are applied from an aqueous slurry of the solid adsorbent with a commercial spreader. The plates are allowed to air-dry for 30 minutes in a horizontal position. They are then activated in an oven at 110° C. for 2 to 3 hours. After activation the plates may be stored in a desiccator until needed.

A mixture of lactic, pyruvic, levulinic, acetic, formic, valeric, propionic, and butyric acids may be separated on silica gel plates using a 1:2 mixture of pyridine/toluene as the developing solvent. The acid solutions are applied 2 cm. from one of the edges of the plate and each spot is confined to as small an area as possible. In cases of dilute solutions multiple spotting of 2 microliters each should be used allowing the spots to dry between applications. The plates are then carefully placed in a glass chromatography tank whose atmosphere has previously been equilibrated with the developing solvent.

After the solvent front has travelled to a height of 10 cm. above the original point of application, the plate is removed and allowed to dry for about 5 minutes. It is then sprayed with bromcresol purple reagent. The acids appear as yellow spots on a blue background.

Better resolution of propionic, valeric, and butyric acids are obtained by deactivating the plate somewhat by exposure to the atmosphere for 30 minutes prior to developing. The method may be used for acids over a large range of concentrations without any appreciable "tailing".

The thickness of the layer is best controlled by the use of a commercial spreader. Layers of varying thickness do not appreciably affect the separation, but they do give erratic Rf values and some sensitivity is lost with thicker layers. The use of a reference acid as a standard against which the migrations of other acids may be compared gives more reproducible results than the conventional Rf values because of variations due to layer thickness. Formic acid is used as a convenient "internal standard" in the eluate during the early portion of the run. Later, when the high formic acid concentrations emerge from the column, we can chromatograph the tube after the formic acid has been evaporated and the residue was dissolved in 70 percent isopropyl alcohol.

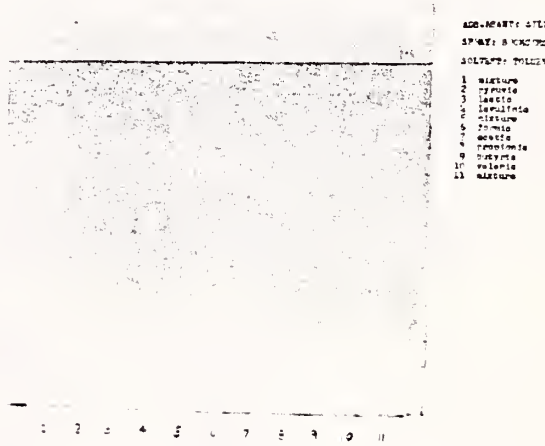

Figure 4.--Separation of liquid acids on silica-gel.

Separation of Solid Organic Acids

The adsorbent used for the separation of the solid acids is cellulose. The layers are prepared in the same way as the silica gel layers. The solvent system used is isobutanol, water, and 9N formic acid in the ratio of 67:78:22, respectively. The plates are pretreated by placing them in the developing tank prior to sample application and allowing the solvent front to travel to the top edge of the plate. The plate is then removed from the tank and dried at 120° C for about 1 hour.

After the plates have cooled to ambient temperature, the acid solutions are spotted 2 cm. from one edge of the plate. The samples should be applied to the edge that rested on the bottom of the developing chamber during the pretreatment step in order to provide for the solvent to develop in the same direction on the plate as previously.

When the solvent front has travelled to 10 cm., the plates are removed from the tank and dried in an oven at 120° C. for 45 minutes to evaporate the formic acid. The plate is permitted to cool and is then sprayed with a 0. 2 percent solution of 2, 6 dichlorophenol indophenol (21) in ethanol. After further heating, the acids appear as white or pink spots on a blue background.

Oxalic, trans-aconitic, cis-aconitic, malic, malonic, tartaric, citric, oxalacetic, alpha-ketoglutaric, fumaric, succinic, and several other acids may be separated from each other by this procedure. Care must be taken to keep the developing chamber saturated. The Rf values are more consistent than the silica gel method, but the time required for development is about 1 hour (4 times longer).

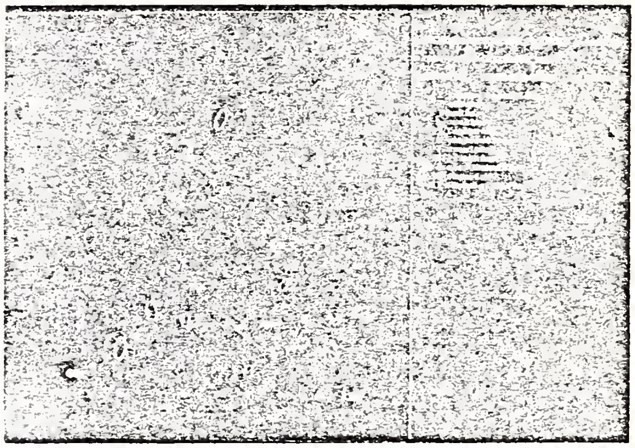

Figure 5.--Separation of solid acids on cellulose.

Results and Discussions

Figures 6, 7, and 8 are indicative of the type of separation obtained with mixtures of known acids. Table 1 summarizes some of the variables studied and their effect on the separation of the acids. The recoveries reported, represent averages rather than individual runs, and since most of these were done when the method was in its early stages of development, we feel they are conservative.

We found that the separations have been adequate for most purposes, but we feel that

Table 1. --Approximate peak elution volumes of organic acids (ml.) at various column heights and gradients

Acid	Column heights (cm.)				Percent Recovery
	5. 5	25	35	25	
	Gr. I	Gr. I	Gr. II	Gr. III	
Aspartic	10	13	--	7	101
Lactic	16	19	40	14	97
Levulinic	17	29	50	22	94
Butyric	20	61	58	50	95
Succinic	32	101	--	76	96
Aconitic (trans)	--	144	77	110	93
Malic	44	149	80	114	99
Propionic	48	171	120	130	87
Tartaric	84	179	---	134	100
Pyruvic	87	188	149	146	93
Citric	100	244	158	176	101
Malonic	114	270	---	204	104
Acetic	126	202	170	213	90
Fumaric	154	330	175	250	99
Aconitic (cis)	171	342	182	261	89
Maleic	---	---	---	278	101

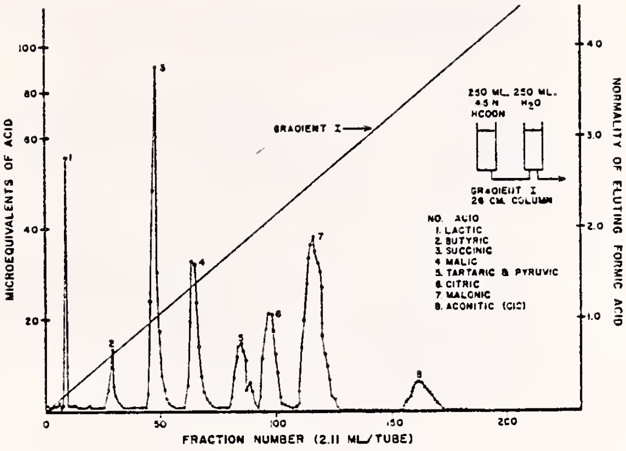

Figure 6.--Separation of eight known acids.

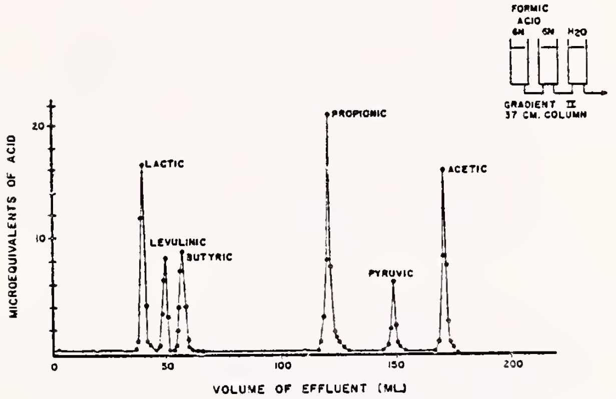

Figure 7.--Separation of six known acids.

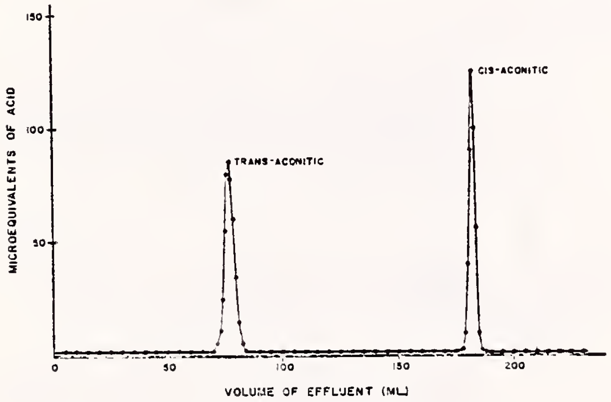

Figure 8.--Separation of cis- and trans-aconitic acid.

the resolution can still be optimized further. For example--only recently, we have become aware of the relative importance of the effect of the flow rate on the degree of separation, with the faster flow rates favoring improved resolution. A systematic study of different flow rates and their effects, in combination with those of column height, normality of eluting acid and type of gradient should improve the method even more.

In our coulometric titration we presuppose that only acidic substances are being titrated at the cathode. If, for some reason, an oxidant is retained on the formate column during the water wash period but is eluted later by the formic acid eluant, it would be titrated as if it were an acid. A method for the external generation of base has been reported in the literature (22 23). The base, which is produced from the electrode decomposition of water, is the same as the one occurring in our present system of internal generation and was given in equation 2.

$$2H_2O + 2e^- \longrightarrow 2OH^- + H_2\uparrow \quad (2)$$

The same circuitry and detection system presently used will be applicable, only the titration will be performed directly in the test tube. This fact will also eliminate the need for quantitatively transferring the contents of the test tube to a beaker for the titration.

Applications

We have not as yet had an opportunity to analyze a large cross section of the refinery materials for organic acid content. However, some of the organic acid profiles we ran are presented in Figures 9, 10, 11, and 12. Figure 9 shows aconitic acid to be the principal acid in this Louisiana raw sugar, accounting for about one-third of the total acid content. The

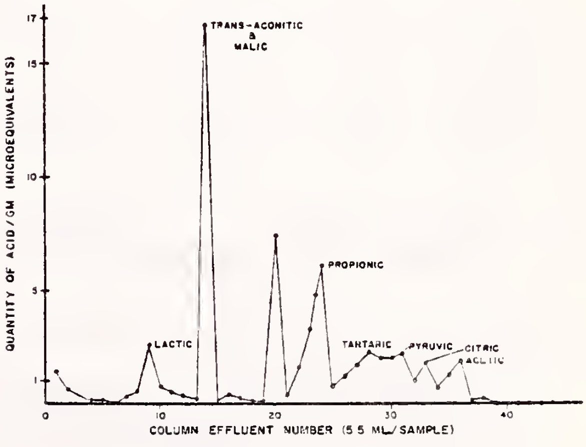

Figure 9.--Elution profile of Louisiana raw sugar--Gradient II.

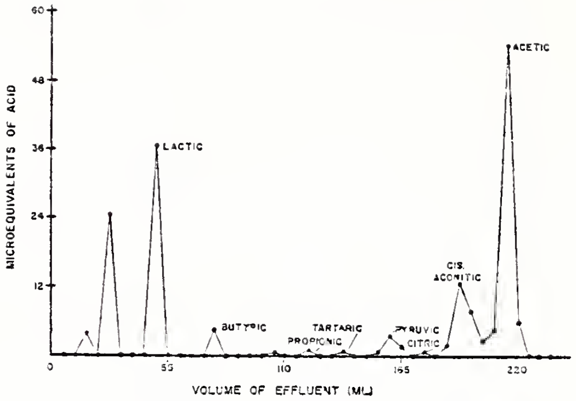

Figure 10.--Elution profile of sweetwater 4.0 ml. Gradient II.

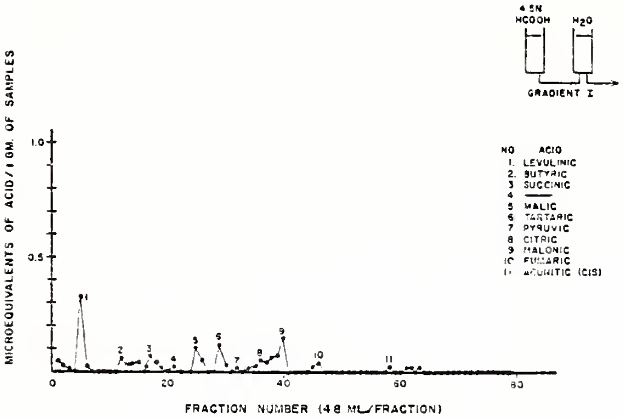

Figure 11.--Elution profile of liquid sugar-- 1 g.

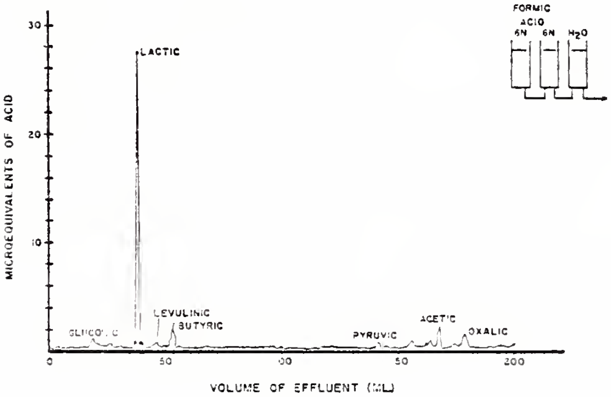

Figure 12.--Elution profile of Bacillus stearothermophilus.

high propionic acid content is probably unusual, but we have found that lactic pyruvic, and acetic acids were common to several raw sugars.

Figure 10 is an acid profile of an ancient refinery sweetwater and is indicative of the problems encountered with materials of this type.

Figure 11 is of interest because it demonstrates the sensitivity of the method. Only 5 g. of samples were used for this analysis of a liquid sugar.

Figure 12 shows how this technique can be useful in microbial taxonomy. The acids found were metabolic products from a pure culture of Bacillus stearothermophilus in 10 percent affination syrup.

Summary and Conclusions

We have presented a method for the determination of organic acids in commercial sugars. The acids are separated on anion exchange resin in the formate form using a formic acid gradient. The effluent is collected in an automatic fraction collector and evaporated to dryness, using a chloroform-formic acid azeotrope as a means of removing the formic acid eluant. The resultant acid residue is titrated coulometrically.

We believe the method presented is reliable, has good resolution, and is sufficiently sensitive to be used for granulated sugar and, at the same time, of sufficient capacity for blackstrap molasses.

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DISCUSSION

N. H. Smith (C&H): Have you found any of these fractions to be colored?

S. Borodkin (American): Yes, this might be a good method for separation. We find that a good portion of the color comes out in the water wash. Some color comes out during the formic acid elution. We find considerable amounts of color coming out in about the fourth or fifth tube prior to where lactic acid emerges. It's also accompanied by a relatively high titration value. Some of the color stays on the column, and it can be washed free from the column with the sodium chloride, so you can break down the color into at least three different fractions.

J. Dowling (Refined Syrups and Sugars): The formic acid that you used, did you redistill it? Because I am sure that the impurities in commercial formic acid are probably as high as some of the acids that you measure.

S. Borodkin: No, we use the analytical grade formic acid, with no treatment at all. We have run the formic acid by the thin layer chromatography and we find it to be pure. We never found traces of any acid but formic acid in the analytical reagent grade.

THE SIGNIFICANCE OF THE LACTATE FERMENTING ANAEROBIC BACTERIA IN SUGAR REFINING

PART I.

THE ISOLATION AND IDENTIFICATION OF A LACTATE FERMENTING BACTERIA COMMONLY FOUND IN RAW SUGAR AND COMMERCIAL CORN STARCH

by
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(Presented by A. B. Rizzuto)

This study is the first in a series of studies delineating the role played by microorganisms in cane sugar refining. To date only two anaerobic sporeforming rods have warranted the attention of the refiners, Clostridium nigrificans and C. thermosaccharolyticum. These studies describe another potentially significant Clostridium capable of fermenting lactate into butyric acid. This investigation concerns itself with techniques involved in isolating and identifying C. butyricum and demonstrating its frequency of distribution in raw sugar and commercial corn starch. Preliminary studies in the refining process present evidence supporting the possibility of significant butyric acid production due to microbial fermentations.

Introduction

In 1927 Werkman and Weaver first identified C. nigrificans as the organism responsible for the sporadic and costly losses from a blackening of canned sweet corn. This is known in the trade as "sulphur stinkers" spoilage. The organism was characterized as a thermophilic sporulating heat-resistant anaerobe. The condition was characterized by the progressive development of a marked blackening of the kernels and the evolution of hydrogen sulfide gas. Later in 1934, McClung described the organism C. thermosaccharolyticum as the organism responsible for the type of spoilage in nonacid canned foods known as "hard swells." This organism was characterized to be strictly anaerobic with an optimum temperature of growth at 55° to 60° C. The organism was further found to be nonproteolytic, extensively saccharolytic, and nonpathogenic. In the development of microbial specification for "Canners'" grade sugar certain limitations were placed on the presence of these organisms in sugar by the National Canners' Association. Since that time the refiner has limited his consideration of the genus Clostridium to these two species. Today "Canners' " grade sugar can be produced routinely by the refiner virtually free of these two troublesome organisms.

In later years "Bottlers' " grade sugars were introduced by the refiners meeting certain microbial specifications concerning yeasts, molds, and aerobic mesophilic bacteria. In addition to the microbial specifications on "Bottlers' " grade sugars a taste and odor limitation was established. It stated that "Bottlers' " grade sugar shall have no obviously objectionable taste or odor in either dry form or in a 10 percent sugar solution prepared with tasteless, odorless water. Today "Bottlers' " grade sugars can be produced routinely, meeting these and all other specifications originally established for this product. Through the years, however, organoleptic controls on the odor quality of certain sugars indicated the occasional presence of an objectionable odor attributed to trace quantities of butyric acid. This acid produces a characteristic odor detectable by human olfactory system in quantities as small as one part per million. Due to its highly offensive characteristic odor and its detectability in trace quantities, the mechanism by which butyric acid is produced in the refining process is of some economical significance.

In 1936, van Beynum and Pette were the first to attribute the butyric acid associated with spoiled grass silage to the lactate fermenting activity of C. tyrobutyricum. Since that time, Martos (1941), Rosenberger (1951), and Bryant and Burkey (1952) have continued to isolate these lactate fermenting sporeforming anaerobes from silage. In 1955, Bryant and Burkey not only confirmed the findings of van Beynum and Pette but also described additional characteristics of these organisms. Bhat and Barker (1947) showed that the fermentation of lactate by a sporeforming anaerobe from soil was dependent on the presence of acetate in the substrate.

The objective of this study is to determine within the refining process the agent or agents responsible for the production of butyric acid, study its mechanism of action, determine its primary sites of origin, and use this information to prevent trace quantities of this acid from further contaminating refined sugar products.

The first series of studies deal with the delineation of certain microbes and the possible role they may play in the production of butyric acid in the refining process. This report in particular will present evidence of the presence of certain anaerobic microorganisms commonly found in raw sugar and corn starch which, if allowed to metabolize, may significantly contribute to the overall butyric acid odor problem.

Materials and Methods

A total of 161 samples were examined for the presence of a lactate fermenting, Gram positive anaerobic sporeforming bacterial rod. Of the 161 samples studied, 92 samples represented raw sugars produced by 10 Louisiana raw sugar mills during the 1963 campaign, 49 samples represented raw sugars produced by various raw mills in 9 foreign countries, and the remaining 20 samples represent corn starch produced by 4 major suppliers.

Isolation Procedure

Three grams of the test material was aseptically innoculated into a large test tube containing 30 ml. of sterile tomato juice broth. The test tube and its contents were shaken until all the sugar was dissolved and then incubated for 72 hours at 37° C. The medium contained trypticase, 3 g.; yeast extract, 3 g.; glucose, 0.3 g.; calcium carbonate, 1.2 g.; cysteine - HCl, 0.15 g.; tomato juice, 60 ml.; sodium thioglycollate, 0.6 g. and distilled water to make 300 ml. After autoclaving for 20 minutes at 121° C. the sterile medium had a pH of 6.5.

After 72 hours' incubation, all tubes were recorded for growth and gas production.

From all tubes showing gas in tomato juice broth 0. 1 ml. was spread plated on tomato juice agar utilizing the Bray anaerobic dish. The Bray dishes were returned to the 37° C. incubator for 48 hours. This medium was the same as tomato juice broth except that 1. 5 percent agar was added and sodium thioglycollate omitted.

Representative isolates were picked from these plates to a modified Bryant and Burkey lactate medium (1956) and incubated for 7 days at 37° C. This medium contained trypticase, 1. 5 g. ; yeast extract, 0. 5 g. ; cystein HCl, 0. 05 g. ; lactic acid, 0. 5 g. ; sodium acetate, 0. 5 g. ; sodium thioglycollate, 0. 2 g. ; and distilled water to 100 ml. The pH was adjusted to 7. 0 and autoclaved for 15 minutes at 121° C. After 7 days' incubation, the pH was determined by glass electrode pH meter and the presence of butyric acid determined by thin layer chromatography.

From all tubes showing a pH rise and butyric acid production 0. 1 ml. was streak plated on tomato juice agar employing the Bray anaerobic dish and incubated for 24 hours at 37° C. A Gram stain of the 24-hour isolates was made and then restreaked on spore medium and incubated anaerobically for 4 days at 37° C. Developing colonies were picked after 4 days incubation and stained for the presence of spores.

Taxonomy Procedure

Thirty-nine representative cultures isolated from the 161 test samples were identified according to Burgey's Manual of Determinative Bacteriology (7 ed.). All cultures used in the taxonomy procedure were Gram positive anaerobic sporeforming rods capable of fermenting lactate.

Results and Discussion

In raw sugar the total anaerobic population is generally lower than the total aerobic population. Any efforts to single out a particular group of organisms in such a widely mixed population invariably requires the use of several microbial techniques. In working with sporeforming anaerobes most investigators use a heat treatment in an attempt to obtain a more restricted microbial population. The danger in this technique is that the spore population of the anaerobe in question may be extremely small or nonexistent resulting in negative information. The method employed in these studies therefore eliminated the heat treatment since only the presence of a particular group of organisms was important and not their actual population count.

Isolation Analysis

The use of tomato juice broth with added sodium thioglycollate served as the initial isolation medium. This medium inhibited strict aerobic growth while enhancing growth of the anaerobes present in raw sugar. The particular nutrients in tomato-juice broth further enhanced the growth of a limited group of anaerobes. Further selection was achieved by restricting the study only to those tubes showing gas production in 72 hours. The use of the Bray anaerobic dish and the spread plating of 0. 1 ml. from a positive tomato juice broth tube cultivated a strict anaerobic isolate capable of maximum growth in the initial isolation medium.

In most cases the colonies developed on tomato-juice agar presented similar colonial morphology. Representative colonies from each plate were picked into lactate broth. This medium restricted growth to organisms capable of fermenting lactate. A positive test in this medium required a pH rise from 0. 5 to 2. 5 pH units accompanied by the production of butyric acid.

The data obtained using the above techniques in the manner previously described are presented in tables 1, 2, and 3.

An analysis of the data presented in these tables indicates the presence of a lactate fermenting anaerobe in 41 percent of the Louisiana raw sugars, 32 percent of the foreign raw sugars, and 100 percent of the corn starches studied. No special significance can be attached to the distribution percentages of these organisms in any particular raw sugar, mainland or foreign, because of the small number of samples evaluated to date. The data do show, however, that these organisms are widely distributed in raw sugar and routinely found in corn starch.

Table 1.--Distribution of lactate fermenting anaerobes in Louisiana raw sugar

Number of samples tested	Mill origin	Tomato juice broth	Tomato juice agar	Lactate broth	
		Gas production	Growth	pH rise	Butyric acid production
11	Enterprise	5	5	5	5
11	Evans Hall	8	8	4	4
11	Glenwood	4	4	4	4
11	Sterling	5	4	3	3
10	Smithfield	4	4	4	4
9	Cinclare	4	4	4	4
9	Caldwell	4	4	4	4
8	Louisia	8	6	6	6
6	Catherine	6	3	1	1
6	Columbia	4	4	3	3
Totals 92		52	46	38	38

Table 2.--Distribution of lactate fermenting anaerobes in foreign raw sugar

Number of samples tested	Country origin	Tomato juice broth	Tomato juice agar	Lactate broth	
		Gas production	Growth	pH rise	Butyric acid production
17	Philippine Islands	13	13	5	5
9	Peru	6	3	3	3
7	Mexico	6	6	3	3
6	Puerto Rico	2	1	1	1
5	Dominican Republic	4	4	2	2
2	Brazil	2	0	0	0
1	Cuba	1	1	1	1
1	Taiwan	0	0	0	0
1	Argentina	1	1	1	1
Totals 49		35	29	16	16

Table 3.--Distribution of lactate fermenting anaerobes in corn starch

Number of samples tested	Supplier	Tomato juice broth	Tomato juice agar	Lactate broth	
		Gas production	Growth	pH rise	Butyric acid production
15	A	15	15	15	15
2	B	2	2	2	2
2	C	2	2	2	2
1	D	1	1	1	1
Totals 20		20	20	20	20

Taxonomy Analysis

Morphological characteristics. --Under the compound microscope at 1250 power these Gram positive organisms appeared as straight or slightly curved rods measuring 0.7 microns wide and 5.0 to 7.0 microns long. These cells were found to occur singly or in pairs with rounded ends. The spores were oval and eccentric to subterminal, swelling the cells to clostridial forms. (Figure 1)

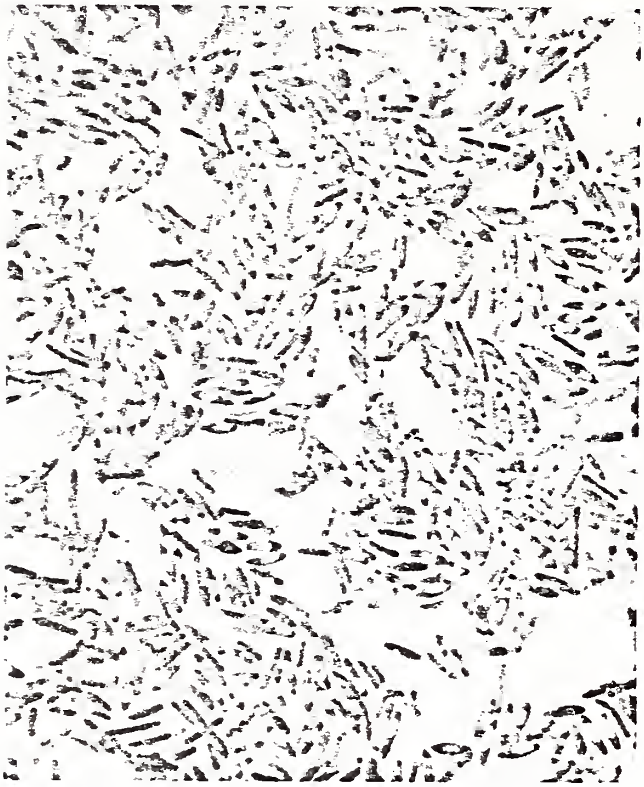

Figure 1.--Spore morphology of Clostridium butyricum at 1,250 magnification.

Cultural characteristics. --Surface colonies grown on tomato-juice agar under anaerobic conditions produced by the Bray dish appeared to be circular, slightly raised, moist and creamy white. (Figure 2)

Physiological characteristics. --In the litmus milk test, 87 percent of the cultures studied produced acid and early coagulation, showing typical stormy fermentation. All cultures showed negative results on indole production and liquefaction of gelatin. Seventy percent of the cultures were not able to produce nitrites from nitrates while 77 percent showed a weakly positive test for hydrogen sulfide production. Of the 20 carbohydrate fermentation tests studied 85 percent of the carbohydrates were fermented by most of the cultures. Louisiana raw sugars showed the most consistent carbohydrate fermentation pattern while the pattern presented in corn starch appeared relatively irregular. The three sugars which most of the cultures could not attack were dulcitol, rhamnose, and inulin. (table 4).

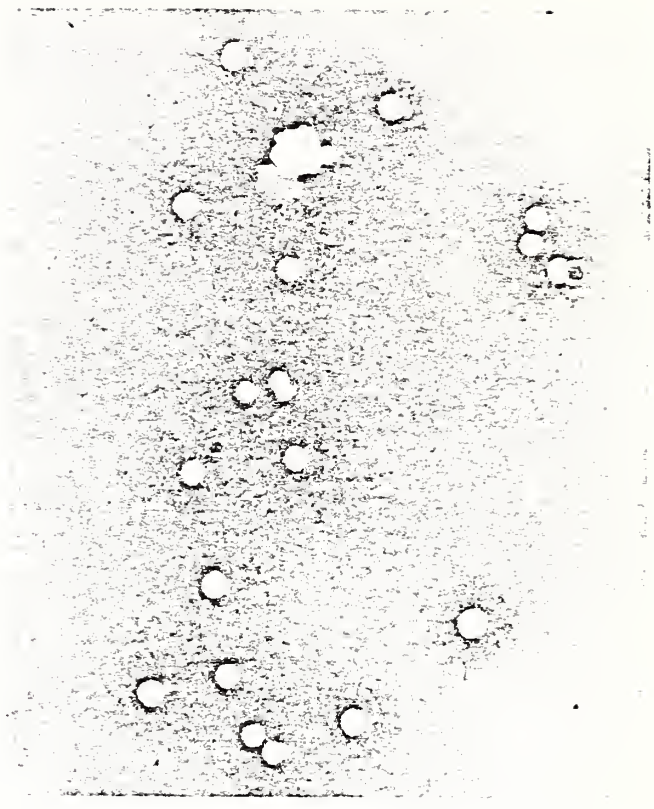

Figure 2.--Colonial morphology of Clostridium butyricum cultivated on tomato juice agar.

There are 93 recognized species within the genus Clostridium. The current edition of Bergey's Manual (7 ed.) recognizes only Clostridium tyrobutyricium and C. lacto-acetophilum as capable of fermenting lactate. More recently the fermentation of lactate was shown to be dependent upon the presence of acetate in the medium. Soon after these findings were reported (4) C. butyricium cultures obtained from the American Type Culture Collection (ATCC) were reported to ferment lactate in the presence of acetate. Bryant and Burkey (1956) claimed their findings invalidated the species Clostridium

Table 4.--Physiological characteristics of lactate fermenting anaerobes

Number of strains	Xylose	Galactose	Maltose	Lactose	Sucrose	Raffinose	Salicin	Inulin	Starch	Mannitol	Glycerol	Lactate	Glucose	Fructose	Arabinose	Dextrin	Mannose	Dulcitol	Sorbitol	Rhamnose	Source	Species designation
12	+	+	+	+	+	±	+	+	+	±	+	+	+	+	+	+	+	-	±	-	Louisiana raw sugar	*Clostridium butyricum*
21	+	+	+	+	+	+	+	-	+	±	±	±	+	+	±	+	+	-	±	-	Foreign raw sugar	*Clostridium butyricum*
6	+	±	+	+	+	+	+	±	+	±	+	±	+	+	+	±	+	±	±	±	Corn starch	*Clostridium butyricum*

lacto-acetophilum which was described as differing from *C. butyricum* only in the fermentation of lactate. They further claimed that *C. tyrobutyricum* should be considered distinct from *C. butyricum* because of the large difference in carbohydrates fermented.

Our findings present evidence of a lactate fermenting anaerobe, which is variable in its ability to reduce nitrates as is *Clostridium tyrobutyricum*, which produces slight amounts of hydrogen sulfide as does *C. lacto-acetophilum* but which strongly resembles *C. butyricum* in all other characteristics. Inasmuch as the carbohydrate fermentation of this organism resembled both *C. lacto-acetophilum* and *C. butyricum* but not *C. tyrobutyricum*, we chose to place it in the type species of the genus, *C. butyricum*.

Preliminary Refinery Studies

Studies on various in-process liquors were conducted to evaluate the cane sugar refining process as a suitable environment capable of supporting the production of butyric acid through microbial action.

A microbial survey of various refinery in-process streams indicated 85 percent of the in-process liquors studies were contaminated with a lactate fermenting anaerobe. The wide distribution of these sporeforming anaerobes in the refinery is a measure of the survival capabilities of this organism as well as the inability of the process to eliminate them.

Since the presence of lactic and acetic acids is essential in the production of butyric acid by the lactate fermentation pathway, various raw sugars and in-process liquors were evaluated for organic acids. Both lactic and acetic acids were repeatedly found in most of the samples tested. Further evidence indicated that although raw sugar routinely contained both lactic and acetic acid, concentrations of these acids appeared to increase in certain in-process streams. On occasions where the lactic acid concentrations were high the microbiology of these liquors usually presented a correspondingly high "flat sour" population . These organisms were isolated and later identified as *Bacillus Stearothermophilus*. When a pure culture of *B. Stearothermophilus* was grown in diluted *in-process liquor*, the resulting organic acid profile showed this organism's ability to produce lactic acid as its major acid and acetic acid as one of its minor acids.

In 1947, Bhat, and others reported that only pyruvates could substitute for acetate in favoring both growth and lactate decomposition by *Clostridium lacto-acetophilum*; therefore, the presence of pyruvic acid was also investigated in the process. Although it was rarely found in relatively large quantities, pyruvic acid was present in almost all in-process liquors tested.

Summary

From these studies it is concluded that certain butyric acid producing anaerobes are common contaminants of raw sugar and commercial corn starch. A microbial survey of various in-process streams indicates the presence of these organisms throughout the refining process. Their presence in the process reflects the organisms' survival capabilities as well as the inability of the process environment to eliminate them. Of the three possible lactate fermenting anaerobes reported in the literature to date, raw sugar and corn starch appear to be principally contaminated with Clostridium butyricum. Corn starch was the most grossly contaminated raw material examined.

The presence of lactic, acetic, and pyruvic acid in the process provides the nutritional requirements necessary for these Clostridia to produce butyric acid using the lactate fermentation pathway. The production of butyric acid in the process by the lactate fermentation pathway appears to be a joint effort between lactic and acetic acid producing microbes and the isolated Clostridium capable of converting the lactic to butyric acid in the presence of the acetate.

The literature has reported similar microbial conversions as the responsible agent in the butyric acid spoilage of grass silage, green Spanish olives, and late gas defects in cheese.

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DISCUSSION

W.A. Bemis (Revere): Mr. Rizzuto, could you tell me the thermal death temperatures of this clostridium; that is, are they thermophiles?

A. B. Rizzuto (American): Mr. Bemis you have asked two questions, may I answer them in the order in which you asked them?

(a) With regard to the thermal death temperatures--at present we have not developed a thermal death curve on this particular organism.

(b) With respect to thermophiles--we would classify this organism as a mesophile since it grew best between 30°and 37° C. We do not mean to imply that it cannot grow at 55° C.

W. A. Bemis: Would they be thermophiles in the spore stage?

A. B. Rizzuto: We use the word thermophile in reference to an actively growing cell; spores are considered the dormant state of most organisms and as such are not generally referred to as either mesophilic or thermophilic.

W. A. Bemis: In the vegetative state, are these clostridia generally the form of a rod shape of bacteria and only in the spore state are they in a clostridia? Could you recognize them in the vegetative stage as bacteria or clostridia?

A. B. Rizzuto: In a vegetative state this organism presents typical rod shape morphology. In the spore state, however, characteristic spores of this organism are oval, eccentric to subterminal, swelling the cells to clostridal forms as demonstrated in Figure 1 of the presentation.

P. F. Meads (C&H): Were these corn starch samples bleached or unbleached?

A. B. Rizzuto: They were both.

P. F. Meads: How does this organism in corn starch affect the refined sugar used for canners?

A. B. Rizzuto: The organism can contaminate the process by entering the refinery either by way of raw sugar or by way of corn starch through the remelt boiling system. Its primary effect on finished products is butyric type odors under certain conditions.

E. P. Barrett (Baugh Chemical Co.): There is one point that I don't understand, the organism both produces butyric acid and increases the pH?

A. B. Rizzuto: One of the peculiar characteristics of this organism as demonstrated by several investigations is the fact that in growing this organism produces excessive amounts of volatile base in addition to butyric acid. The sum total of this metabolism always produces a detectable rise in pH.

J. B. Stark (WURDD): How much is this pH rise?

A. B. Rizzuto: It would go up about five tenths of a pH and can sometimes get a little bit higher. It's very characteristic of this particular species, or at least three of them.

W. A. Clarno: How effective is good filterability in the filter station in removing these clostridia?

A. B. Rizzuto: Good tight filtration, using diatomaceous earth can be an effective tool in removing most of these organisms from the filtered liquor; however, absolute removal is questionable routinely on a practical basis. The number of organisms potentially present in the filtered liquor involves several factors, one important factor being the organism population in the liquor prior to press filtration.

S. Stachenko: (C&D): Do you have any data on wheat starch?

A. B. Rizzuto: No, I do not.

PROGRESS IN RESEARCH ON THE SOLUBLE POLYSACCHARIDES OF SUGARCANE

by
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(Presented by E. J. Roberts)

Some of the organic nonsugars of cane juice are known to have adverse effects on clarification and crystallization processes during sugar manufacture. Small amounts of these compounds are carried through into the raw sugar by adsorption and occlusion in the crystals and then become problems for the refiner. Our research, for the past several years, has been concerned with the identification and determination of these organic nonsugars in sugarcane products. This work is part of our overall research program to improve the yield and quality of raw sugar through the development of new and more efficient clarification processes. Reports of some of this work have been presented at the Technical Sessions of the Bone Char Research Project (1). The polysaccharides are the most abundant of the organic nonsugars in cane juice other than the organic and amino acids. Starch, which is one component of the polysaccharides complex, has been studied extensively. The soluble polysaccharides, which are several times the concentration of starch, have not been studied extensively and their effects on sugar manufacturing and refining are almost completely unknown. Early workers referred to these polysaccharides as, "gums," and assumed that they were composed of arabans, xylans, and pectin (2, 3). They were chiefly concerned with developing methods for measuring the quantities of these polysaccharides present in sugarcane products. Ruff and Withrow (4) developed a quantitative method in which the polysaccharides were precipitated by alcohol from settled juice, the precipitate was filtered off on a Gooch crucible, dried, weighed, and ashed. The loss on ignition was taken as the weight of the polysaccharide. Fort and McKaig (5) employed an improvement of this method in which the polysaccharides were precipitated with alcohol, the precipitate was filtered off on a mat of filter aid and the sugars were washed out of the mat with 75-percent aqueous alcohol. The filter aid mat was digested in hydrochloric acid to hydrolyze the polysaccharides and the resulting sugars were determined by copper reduction. This method included starch, as well as the soluble polysaccharides, and was subject to error due to the fact that some reducing sugars are destroyed in hot acid solution.

In 1958, Nicholson and Lilienthal (6) isolated a polysaccharide fraction from juice of spoiled cane, by alcohol precipitation, which was similar to dextran in physical and chemical properties. They concluded that this polysaccharide was produced by the action of micro-organisms on the sugar during the delay between cutting and milling the cane. In 1960, Sutherland (7) reported the isolation of two polysaccharides from sirups. They were separated by fractional precipitation with alcohol and one of the polysaccharides was found in all samples examined and appeared to be of the hemicellulose type. The other polysaccharide was a poly-glucose of the dextran type, and its presence was associated with large increases in the viscosity of the sirups. Sutherland found that all of the fractions yielded glucose, galactose, arabinose, xylose, and rhamnose upon hydrolysis and the specific rotation of the fractions ranged from +57° to +198°. In this report we present the methods used in the isolation of sugarcane polysaccharides from molasses and juice and some of the properties of the isolated polysaccharides and methods for their quantitative determination in sugarcane products.

Isolation of Polysaccharides

Molasses and mixed juice polysaccharides.--The polysaccharides were isolated, by alcohol precipitation, from several lots of normal commercial final molasses from different factories and refineries. The molasses was first diluted to about 50° Brix with water and sufficient alcohol was added to produce an alcohol concentration of 75 percent by volume. The precipitate was allowed to settle for several hours, the supernatant liquid was decanted, the precipitate was suspended in water by stirring, and the insoluble material was removed by centrifuging or by filtration through a mat of filter aid. The polysaccharide was again precipitated from the filtrate by adding alcohol to produce an alcohol concentration of 75 percent. A few ml. of acetic acid was added to aid in coagulating the precipitate. After the precipitate settled for several hours it was filtered off, redissolved in water and dialyzed in 1-1/8 inch diameter cellophane bags against flowing toluene-saturated water for 60 hours. When the dialysis was complete the solution in the bags was passed through a column of DEAE cellulose, to remove color, and was centrifuged at 18,000 r.p.m., 40,000 G., to remove most of the turbidity. The polysaccharide was again precipitated as previously described. After the precipitate had settled it was filtered off with suction, washed several times with absolute alcohol and then with ether, and dried in the air at room temperature.

The cane juice used for the isolation of juice polysaccharide was reconstituted from mixed juice solids obtained in our pilot plant at the Audubon Sugar Factory in 1963 and preserved by freeze drying as described by Roberts and Martin (1). The polysaccharide was isolated from a 15 percent solution of juice solids by the procedure described for molasses except that 1 g. of filter aid was added for each 100 g. of juice used and decolorization with DEAE cellulose was not necessary.

A 100 mg. sample of each preparation was hydrolyzed by refluxing in 2 N sulfuric acid for 3 hours. The sulfuric acid was removed by the addition of a slight excess of barium hydroxide and the excess barium was removed by passing carbon dioxide through the solution. The precipitated barium sulfate and carbonate were removed by filtering the solution through a mat of filter aid. The solution was evaporated by blowing a stream of air over the solution at room temperature. The solid material remaining was dissolved in 10 ml. of water and spotted on strips of Whatman 3MM chromatographic paper. The chromatograms were triple-developed, by the ascending technique, with n-butanol-acetic acid-water 4:1:5 v/v and dried. The papers were sprayed on one side with anisidine phosphate which upon heating produced a yellow color with fructose and a brown color with the other sugars (8). The papers were then sprayed on the reverse side with aniline phthalate which upon heating produced a brick-red color with pentoses and a brown color with the other sugars. All of the chromatograms showed the presence of glucose, fructose, arabinose, xylose, rhamnose, fucose, and galactose. This polysaccharide is found in all sugarcane products from raw juice to refined sugar. Partial analysis of molasses and mixed juice polysaccharides are shown in table 1.

Table 1.--Partial analysis of polysaccharides isolated from molasses and mixed juice

Source	$[\alpha]^{20^\circ}_{5461A}$	Glucose (pct.)	Fructose (pct.)
Molasses	+133°	55.4	19.2
Molasses	+161°	57.8	7.1
Molasses	+120°	55.6	9.3
Mixed juice	+115°	50.6	2.6
Mixed juice	+ 38°	36.4	4.7
Mixed juice	+ 66°	37.3	3.8

The specific rotations were determined with the ETL-NPL automatic polarimeter and the glucose determinations were made with the glucostat reagent (9) after hydrolysis of the polysaccharide with 4 N hydrochloric acid by digestion in boiling water for 20 minutes. The fructose was determined colorimetrically with cysteine hydrochloride-sulfuric acid as described by Dische and Devi (10). Work on the determination of the pentoses and galactose is in progress.

When a solution of the polysaccharide was treated with invertase overnight, a small amount of fructose was hydrolyzed off, as shown by paper chromatography, but further treatment with invertase did not produce further hydrolysis. If a solution of the polysaccharide was adjusted to pH 1.5 - 2.0 with sulfuric acid and allowed to stand overnight at room temperature a small amount of fructose was hydrolyzed off. The fructose hydrolyzed off by invertase or by acid treatment at room temperature represented a small fraction of the total fructose in the polysaccharide as the partially hydrolyzed polysaccharide in the invertase treated solution or the acid treated solution could be precipitated at 75-percent alcohol concentration and upon hydrolysis in refluxing 2 N sulfuric acid for 3 hours still yielded fructose, as shown by paper chromatography, along with the other sugars normally found in the hydrolyzed polysaccharide.

In order to determine whether the fructose in the polysaccharide was part of the molecule or whether it was derived from a fructan which was precipitated along with the polysaccharide, a sample was partially hydrolyzed with 0.1 N sulfuric acid at 100° C. for 1 hour. The sulfuric acid was removed as previously described and paper chromatography of the concentrated solution showed the presence of 4 oligosaccharides, with Rf values lower than that of glucose, along with some monosaccharides and a small amount of unhydrolyzed polysaccharide. Each oligosaccharide was eluted from another chromatogram, after location by a guide strip, and completely hydrolyzed. Paper chromatography of the hydrolyzed oligosaccharides showed the presence of glucose, fructose, and traces of pentoses in each. It was, therefore, concluded from these results that fructose is part of a heterogeneous polysaccharide molecule.

Fresh juice polysaccharide.--Chemical compounds elaborated by normal plants usually exhibit constant physical properties and chemical composition from one preparation to another. The variations in the specific rotation and in the glucose and fructose content of the polysaccharides from molasses and mixed juice led us to suspect that all of these products were contaminated with one or more abnormal polysaccharides produced by microorganisms or by abnormal metabolism in the cane during the delay between cutting and milling. In order to ascertain if this was true, a sample of polysaccharide was prepared from cane which was ground as soon as possible after harvesting.

Through the courtesy of Southdown Sugars, Inc., we obtained approximately 2 tons of cane and with the cooperation of Crops Research Division, Houma, La., the cane was cut on September 24, 1963, with a mechanical harvester which never allowed the cane to come in contact with the ground. It was transported about on-half mile to the Houma Station and was ground without maceration. The juice, 305 gallons containing 475 pounds of solids, was pumped into a tank and 14 pounds of filter aid, 950 gallons of absolute alcohol and 35 pounds of acetic acid were added while the solution was stirred mechanically. The time from cutting until the alcohol was added was less than 2 hours. This was the minimum time that was practical with this amount of cane. After settling over night, the supernatant liquid was pumped out and the precipitate was filtered off on a plate and frame filter. The precipitate was placed in a stainless steel can and covered with absolute alcohol to prevent spoilage and was brought back to New Orleans where the polysaccharide was recovered from 1,000 g. batches of the filter cake in the same way as described for mixed juice. The yield of polysaccharide was 16 g. This polysaccharide had a specific rotation of -46.2° at 20° C. The polysaccharide isolated from each of 2 quarts of quick frozen fresh cane juice later that year showed similar specific rotations.

In order to confirm these findings another batch of 650 pounds of cane was cut and quickly ground on August 27, 1964, yielding 44 gallons of juice. To this was added 2 pounds of filter aid, 150 gallons of absolute alcohol, and 3 pounds of acetic acid. After settling the supernatant liquid was decanted leaving about 30 gallons of slurry. The bagasse was macerated with two 9-gallon quantities of water yielding 17 gallons of maceration juice. To this was added 1 pound of filter aid, 50 gallons of absolute alcohol, and 1 pound of acetic acid. After settling the supernatant liquid was decanted leaving about 20 gallons of slurry. The slurries were brought to New Orleans, filtered off, and the polysaccharide was recovered from the filter cake as previously described. The crusher juice yielded 8 g. of polysaccharides after some loss during dialysis and the maceration juice yielded 14 g. Partial analysis of these polysaccharide fractions carried out as described for molasses and mixed juice are shown in table 2.

Attempts were made to fractionate the polysaccharide by high voltage paper electrophoresis with an apparatus similar to that described by Gross (11) but were unsuccessful. Attempts were also made to fractionate the

Table 2.--Partial analysis of polysaccharides isolated from fresh juice

Source	$[\alpha]^{20^\circ}_{5461\text{A}}$	Glucose (pct.)	Fructose (pct.)
Crusher juice (1963)	-46.2°	2.73	1.3
Frozen juice (1963)	-45.5°	---	---
Frozen juice (1963)	-47.1°	---	---
Crusher juice (1964)	-45.6°	6.45	1.11
Maceration juice (1964)	-45.0°	7.8	0.75

polysaccharide on Sephadex columns. This was also unsuccessful even on columns of Sephadex G-100 indicating a relatively high-molecular weight.

Determination of Molecular Weight

The molecular weight of the polysaccharide was determined from turbidity measurements. Light scattered at 90° was measured on a Brice-Phoenix instrument at 436 mμ. The dissymmetry was always of the order of 1.5 to 2, which in itself indicated a high molecular weight. The excess scattering at 90° was corrected for dissymmetry by the method of Flory (12) using the tables of Doty and Steiner (13) for polydispersed coils. This correction is positive and incidentally an estimate of the particle size is obtained. Depolarization was found to be always small and was taken as constant at a 3 percent negative correction. Fluorescence was determined to be negligible.

The relationship between molecular weight and corrected turbidity was derived by Debye (14) to be:

$$\frac{32\pi^3n^2}{3\lambda^4N}\left(\frac{dn}{dc}\right)^2\frac{c}{\tau}=\frac{Hc}{\tau}=\frac{1}{M^w}+2\,Bc$$

where c is the concentration in g./ml., τ the corrected excess turbidity of the solute in cm.,$^{-1}$ M^w the weight average molecular weight, g./mole, n the refractive index of the solvent, N Avagadro's number, λ the wavelength of the incident light in cm., dn/dc the refractive index increment of the solute, and B is a constant that is related to interaction between particles. Edsall and others (15) have shown that B is very small for large uncharged particles. The polysaccharide was expected to be in this category and preliminary measurements indicated that indeed c/τ_{90} was independent of c within a few percent for a hundredfold range in c, as shown in Figure 1.

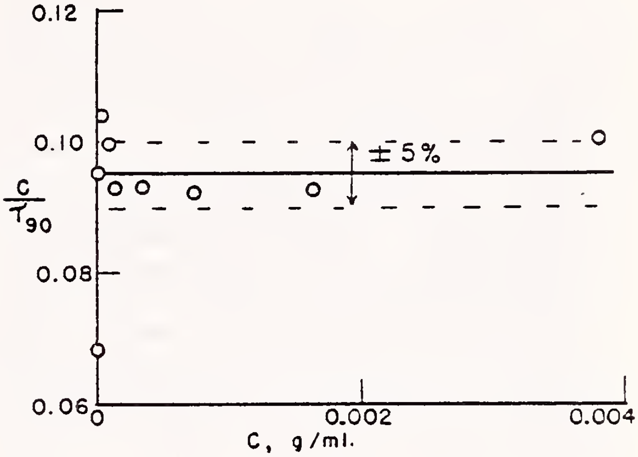

Figure 1.--Turbidity plot for gum, showing independence and concentration.

The last term in the equation was, therefore, neglected and extrapolations to zero concentrations were not required. The refractive index increment was measured on the Phoenix differential refractometer and was found to be, 0.162 ml./g. for all the polysaccharide samples in the range of concentrations used. The concentrations were based on the estimated purity of the polysaccharide preparations by anthrone evaluation of their carbohydrate content. The concentration of filtered solutions was evaluated from the refractive index and from the dilution. The value of the constant H was 7.14×10^{-6}.

The calibration of the instrument was checked by measuring the turbidity of benzene and carbon tetrachloride, as shown in table 3. The entire procedure was checked by measuring the molecular weight of sucrose after the method of Maron and Lou (16). The value found was 459 which is 34 percent higher than the true value, but it is considered a very good check in view of the tremendous difficulty in cleaning up sucrose solutions.

Table 3.--Check of calibration of instrument (4882-34)

Substance	Recognized turbidity	Measured turbidity
Benzene	8.11×10^{-4} cm.$^{-1}$	8.56×10^{-4} cm.$^{-1}$
Carbon tetrachloride	2.57×10^{-4} cm.$^{-1}$	2.68×10^{-4} cm.$^{-1}$

In general, the molecular weights found were of the order of a few million which was larger than had been expected from the chemical nature of the polysaccharide. Attempts to further disperse the polysaccharide were not very successful. Heating, cooling, drastic pH changes, and agitation did not produce significant changes. However, definitely lower molecular weights were obtained by using 1.0 N potassium hydroxide or 20 percent calcium chloride as solvent instead of water. It was also found that filtration through progressively finer membrane filters gave progressively lower average molecular weights as shown in Figure 2.

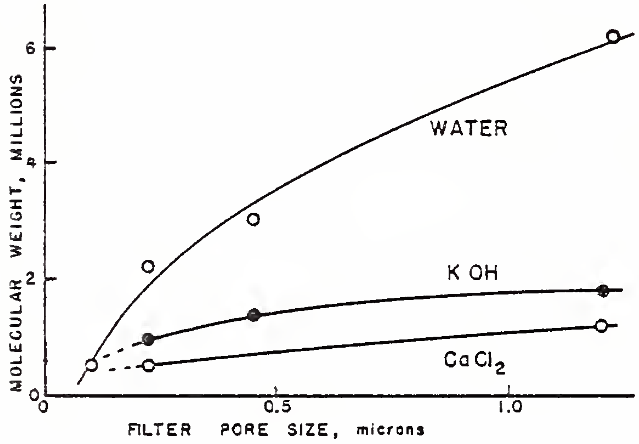

Figure 2.--Effect of solvent and filtration on apparent molecular weight of gum.

The light scattering method determines a weight average molecular weight, which is defined as: $M_w = \frac{\Sigma W_i M_i}{\Sigma W_i}$, where W_i is the weight and M_i is the molecular weight of an individual particle. This average is influenced most by the large particles so that a relatively few large particles would disproportionately increase the average.

From the data in Figure 2 it appears that there are a few incompletely dispersed particles in the water solutions while in potassium hydroxide and calcium chloride the dispersion is more complete. Filtration through a 0.1 micron membrane filter eliminates the undispersed particles in water and gives a molecular weight that is in agreement with data obtained in potassium hydroxide or calcium chloride solutions. Taking the data obtained in water, potassium hydroxide and calcium chloride collectively, it appears that the molecular weight of the polysaccharide as determined by light scattering probably ranges from one-half million to 2 million.

The superior dispersion of the polysaccharide in potassium hydroxide and calcium chloride solutions is similar to the effect of these reagents on starch. Starch, however, has a molecular weight of the order of 10 million to 100 million.

The dissymmetries which were measured to obtain a correction for turbidity also gave an estimate of particle size. The value obtained was always of the order of 0.1 to 0.05 microns, which is in agreement with the size of membrane filters necessary to eliminate undispersed particles. This value is of the order of one-fifth the wavelength of light and the scattering should, therefore, approximate to the Rayleigh law. Angular scattering measurements were made to test this hypothesis and the results are shown in Figure 3 where the angular scattering of the polysaccharide is compared with starch and with Rayleigh scattering. It is the shape of the curve rather than the absolute value that is of consequence. It is seen that the polysaccharide does indeed approach Rayleigh scattering except in the forward direction where the transmitted beam begins to be measured. These data can be interpreted as not inconsistent with a particle size of 0.1 micron and a molecular weight of 1 million.

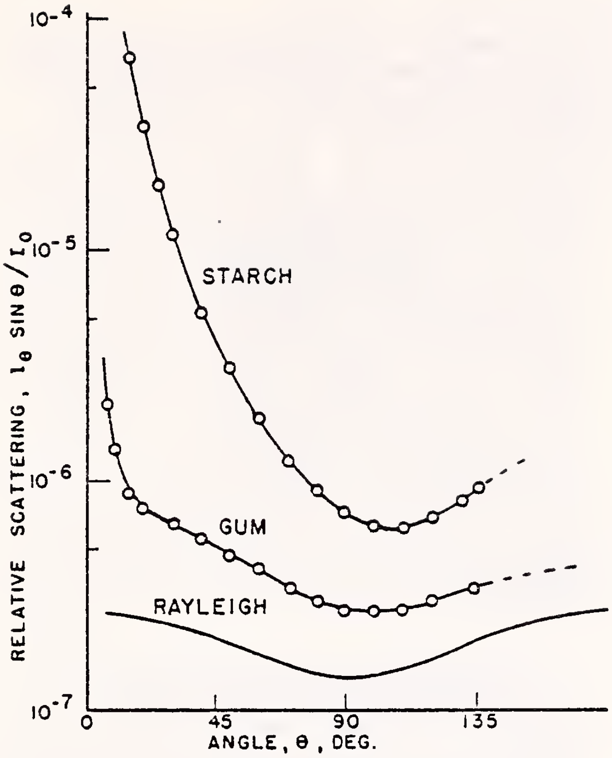

Figure 3.--Scattering envelopes.

An alternate method of determining molecular weight is by osmotic pressure, however the level of 1 million is at the extreme upper limit of the method. A quick check of osmotic pressure of the polysaccharide solution indicated that the molecular weight was high although perhaps only 100,000. Since osomotic pressure measures the number average molecular weight, which in polydispersed systems is always smaller than the weight average, this result is not inconsistant with the values of one-half million to 2 million obtained from light scattering.

Nonprecipitable polysaccharide.--Carbon-celite columns of various types have been used to separate monosaccharides from disaccharides and oligosaccharides as is found in the hydrolysis products of starch and other polysaccharides (17), (18). The possibility of using a carbon-celite column to separate the polysaccharides of sugarcane from the sugars, starch and other constituents and thus serve as a method for measuring the quantity of polysaccharides in sugarcane products was investigated.

One gram of 80° Brix final molasses was dissolved in 10 ml. of water and placed on a carbon-celite column similar to that of Whistler and Durso (17), composed of 5 g. of carbon G-60 and 15 g. of celite 535, and the sugars were eluted with 500 ml. of 10 percent alcohol. The solvent was then changed to 50 percent alcohol and the first 50 ml. of eluate contained no carbohydrates as shown by the phenolsulfuric acid test (19) and was discarded. The following 200 ml. of eluate contained a polysaccharide which had a specific rotation of +33° and upon hydrolysis yielded glucose, fructose, arabinose, xylose, rhamnose, fucose, and galactose. The quantity of the polysaccharide could be determined colorimetrically by the phenolsulfuric acid method (19) and was found in all sugarcane products from raw juice to refined sugar and was assumed to be the same polysaccharide as that obtained by alcohol precipitation. In order to determine whether the polysaccharide could be eluted quantitatively from the carbon-celite column a sample of polysaccharide, obtained from molasses by alcohol precipitation and purified by dialysis, was placed on a column and when eluted with 50 percent alcohol, no polysaccharide was found in the eluate. All efforts to desorb the polysaccharide were unsuccessful.

Another sample of molasses was then placed on the column and the eluted polysaccharide was dialyzed for 60 hours. Examination of the solution remaining in the bag showed the polysaccharide had dialyzed out. It was evident that the polysaccharide that was eluted from the column was not the same as that obtained by alcohol precipitation. The eluted polysaccharide is evidently of relatively low molecular weight, is not precipitated at 75-percent alcohol concentration, and is dialyzable. The polysaccharide was difficult to isolate in quantity and was usually worked with in solution as obtained from the carbon-celite column. A partial analysis of several preparations of the nonprecipitable polysaccharide is shown in table 4.

The conclusion that this polysaccharide was of relatively low molecular weight was substantiated by the facts that it emerged from a Sephadex G-75 column in the third void volume and is separated into three spots of low mobility when subjected to high voltage paper electrophoresis with 0. 1 N sodium hydroxide buffer.

Table 4.--Partial analysis of non-precipitable polysaccharides

Source	$[\alpha]^{20^\circ}_{5461A}$	Glucose (pct.)	Fructose (pct.)
Molasses	+33°	40. 2	14. 0
Mixed juice	+52°	40. 0	13. 0
Mixed juice	+37°	38. 2	12. 4
Mixed juice	+46°	34. 3	11. 1

Analytical Methods

Nonprecipitable polysaccharide.--As mentioned above a nonprecipitable polysaccharide fraction could be separated from the sugars and other materials in sugarcane products by a carbon-celite column and determined with phenol-sulfuric acid reagent. This became the basis of the method for the determination of this polysaccharide fraction.

Preparation of the column.--The columns used were 26 mm. in diameter with a glass wool plug on a perforated porcelain disk to support the adsorbent mixture; a total height of approximately 40 cm. provided for convenient charging and for maintaining the level of solvent about 5 cm. above the top of the adsorbent bed. A mixture of 5 g. of carbon G-60 and 15 g. of celite 535 was suspended in 150 ml. of water and after agitation in a high speed mixer for a few seconds was poured into the tube. The solvent reservoir, containing 200 ml. of 10 percent alcohol and arranged for applying pressure, was connected to the top of the tube and the solvent was forced through the adsorbent under about 10 pounds of air pressure to pack the adsorbent. Addition of a 1 cm. layer of celite above the adsorbent before the solvent was all forced in prevented plugging of the column by suspended matter in the sample.

Application of the sample and elution of the polysaccharide.--Solutions of the materials to be analyzed are made of such concentrations that a 5 to 20 ml. aliquot contains the proper amount of sample to be applied to the column; about 0. 5 g. of molasses solids, 3 g. of juice or sirup solids, and 5 g. of raw sugar. The sample is placed on the column and forced into the adsorbent under about 2 pounds' pressure. The sugars, sucrose and invert, are eluted with 500 ml. of 10 percent alcohol, 750 ml. for raw sugar because of the larger sample, at a flow rate of 4 to 5 ml. per minute. The solvent is then changed to 50 percent alcohol and the first 50 ml. of effluent contains no polysaccharide and is discarded. The next 200 ml. containing all of the polysaccharide fraction is collected in a 200 ml. volumetric flask and 2 ml. of this solution is used for colorimetric determination by phenol-sulfuric acid. The absorbence of the color at 485 mμ is measured and the quantity of polysaccharide is read from a standard curve, prepared from pure glucose in 50 percent alcohol, and expressed as anhydro glucose.

Precipitable polysaccharide.--The alcohol precipitable polysaccharide fraction cannot be desorbed from the carbon column but can be separated from the nonprecipitable polysaccharide, sugars, and salts by dialysis. Such a quantity of material to be analyzed as to contain 10 g. of solids is weighed out, dissolved in 25 ml. of water and poured into 1-1/8" diameter x 18" cellulose dialyzer tubing and rinsed into the tubes quantitatively with an additional 25 ml. of water; when juice is analyzed no additional water is added. The tubes are then tied allowing an air space so that the total volume of tube is less than 100 ml. and the solutions are dialyzed against flowing toluene-saturated water for 60 hours. The tubes are emptied into beakers, rinsed with a little water, acidified with 1 ml. of 1. 0 N sulfuric acid, and diluted to 100 ml. in a volumetric flask. An aliquot of 25-50 ml. of each solution is centrifuged; usually about 20 minutes at 18, 000 r. p. m., 40, 000 g. This removes insoluble starch and other suspended material. An aliquot of the supernatant solution, estimated to contain 1. 0-10. 0 mg. of polysaccharide is diluted to 100 ml. for colorimetric determination. For raw sugar dilution of the centrifuged solution is usually not necessary. The colorimetric determinations are made on 2 ml. of these solutions by the phenol-sulfuric acid reagent and the absorbance of the color at 485mμ is measured and the quantity of polysaccharide is read from a standard curve prepared from pure clusoce in water and expressed as anhydro glucose. Some typical results

Table 5.--Polysaccharides in sugarcane products

Product	Precipitable polysaccharides solids (pct.)	Nonprecipitable polysaccharides solids (pct.)
Mixed raw juice	0.23	0.13
Clarified juice	.25	.13
Raw factory molasses	1.74	1.18
Raw factory molasses	1.11	.93
Raw sugar	.08	--
Washed raw sugar	.04	--
Granulated sugar	.03	--
Affination sirup	.30	--
Refinery molasses	.80	--

obtained by the use of these methods are shown in table 5.

Discussion and Summary

The purpose of our research on the composition of sugarcane juice is to extend our knowledge of the individual juice constituents because only through a thorough knowledge of the nature and quantities of these constituents can intelligent attempts be made to develop new and better clarification and refining processes.

The soluble polysaccharides account for a significant portion of the organic nonsugars of sugarcane juice, being exceeded in concentration only by the amino acids and the organic acids. These polysaccharides have been isolated from molasses, sirup, mixed juice, and juice from freshly cut and quickly ground cane. The juice from freshly cut and quickly ground cane contains a single type of polysaccharide which exhibits a negative specific rotation of constant value and is considered to be the natural polysaccharide of sugarcane. The polysaccharide isolated from all other sugarcane products, including refinery products, seem to contain one or more abnormal polysaccharides produced by microorganisms or by abnormal metabolism of the cane during the delay between cutting and milling. This mixture of polysaccharides has not been separated and exhibits specific rotations ranging from +38° to +161° and molecular weights ranging from one-half million to two million as determined by light scattering measurements. About two-thirds of the polysaccharide complex is alcohol precipitable and is nondialyzable, the other one-third is not alcohol precipitable and is dialyzable. Upon hydrolysis all of the polysaccharides isolated yield glucose, fructose, arabinose, xylose, rhamnose, fucose, and galactose.

Methods have been devised for the quantitative determination of the total soluble polysacchrides in all sugarcane products.

Acknowledgments

We are indebted to our colleagues Messrs. W. F. Guilbeau and Emory E. Coll, who prepared the lyophilized mixed juice samples; to Mr. A. J. Isacks, of Southdown Sugars, Inc., Houma, La. who supplied the samples of fresh cane; and to Dr. E. V. Abbot of Crops Research Division, Houma, La. who allowed us the use of the milling equipment.

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DISCUSSION

S. Borodkin (American): Maybe I didn't get it, but you spoke of the nonalcohol precipitable portion that was dialyzable, and an alcohol precipitable portion that was not dialyzable. Have you done any work with the material that was alcohol precipitated but was dialyzed?

E.J. Roberts (SURDD): We have never found such a fraction but there could be a small amount of material present that falls into this category.

F.M. Exley (Savannah): Has any work been done on trying to locate in the body of the cane, in the rind, or in the joint or in the leaf sheath where the polysaccharides are more numerous?

E.J. Roberts: Not as far as I know. I think that would be a good thing to look at, but we haven't studied that yet.

N.H. Smith (C&H): Have you looked at the effect of temperature on the precipitability of the polysaccharides or the time required for precipitation?

E.J. Roberts: We usually let the precipitate settle overnight, but we have not really studied the effect of temperature or time on the precipitation.

H.G. Gerstner (Colonial): This concludes the Technical Session, but before we adjourn there are some things that have to be expressed. First, our appreciation to the speakers for the time, trouble, and effort put into the papers, to Dr. Carpenter, and to the United States Department of Agriculture, specifically Mrs. Sharar for the efficiency and precision with which this session was run off.

The staff has asked me to tell you that you should begin to think about the next session 2 years hence, which will emphasize constituents in the sugar; for example, floc formers, color, and phosphoric acid defecation. You are encouraged to think about this and be prepared with your presentations.

The meeting is adjourned.

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